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Living in a Plant:
Brain and Behavioral Traits of Acacia Ants

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Living in a Plant:
Brain and Behavioral Traits of Acacia Ants

by

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Dedication

I dedicate this work to my parents, Rodolfo Amador and Sodelba Vargas, for their eternal love and support, and for letting me play with ants during my childhood;
and to William G. Eberhard, whose lessons I will carry with me forever.

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Living in a Plant:
Brain and Behavioral Traits of Acacia Ants

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The University of Texas at Austin, 2014

Supervisor: Ulrich G. Mueller

Acacia ants evolved obligate protective mutualisms with acacia trees, which they defend against herbivores, food parasites and encroaching vegetation. In this mutualism, the fitness of one partner entirely depends on the other. Other ant species are parasitic on acacia trees; they nest on the tree, harvest food rewards, do not defend their own tree, and occasionally try to steal food from other trees, usually inhabited by mutualistic ants. To understand the behavioral and anatomical effects of the interaction between ants and host trees, I integrated brain anatomy, morphology and field experiments to study parasitic and mutualistic species of *Pseudomyrmex* ants associated with acacia trees. In **Chapter 1**, I describe a previously unknown behavior of stealing food from other ant-defended acacia trees in the parasitic acacia ant *P. nigropilosus*, and I evaluate four strategies that may allow parasitic ants to overcome the usually effective defenses of the robbed mutualistic ants protecting a host tree. In **Chapter 2**, I study how colony size correlates with the degree of division of labor and brain anatomy of workers, focusing on a species of acacia ant lacking morphological castes among workers, *P. spinicola*. In **Chapter 3**, I study acacia-ant behavior of killing vegetation encroaching on a host tree. I document the

interspecific differences among acacia ants in the size of the area around the host tree that workers clear from encroaching vegetation. I further test for interspecific variation in pruning behavior, and whether mandibular force correlate with worker pruning decisions. In **Chapter 4**, I test whether ant species that routinely leave the host tree to forage or to prune encroaching vegetation are better at orienting themselves when returning to their host tree, compared to ant species that rarely leave their host tree. This dissertation documents how the obligate protective mutualism of an ant with a tree has consequences for division of labor, navigational skills, behavioral specializations, head shape and brain anatomy of ant workers.

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Introduction

Ant-plant interactions have been the focus of many ecological studies in the past 40 years, elucidating both the proximate and ultimate mechanisms of these interactions (Rico-Gray and Oliveira 2007). Since the pioneer work of Daniel H. Janzen on the coevolution of *Pseudomyrmex* ants with acacia trees (Janzen 1966; Janzen 1974), researchers recognized the usefulness of ant-plant interactions to address ecological, behavioral and evolutionary questions. Also, the sociality of ants that are associated with plants has been rarely studied, despite the advantage of having the entire colony located aboveground and limited to the plant host. For instance, our knowledge is very limited regarding colony organization, division of labor in the colony, individual decision-making and worker specializations related to colony and plant-host needs.

In obligate plant associations, the plant provides the ant colony with food (e.g., nectar, protein-rich food bodies) and safe nesting cavities; hence, the fitness on the ant colony depends largely on the plant (Hanson and Longino 2006; Rico-Gray and Oliveira 2007). Defense of the host plant is therefore a very important aspect of ant fitness engaged in protective mutualisms with plants, especially if the ants are obligate plant associates such as acacia ants. Besides defending the colony against parasites and food robbers, ants that are obligate plant associates also have to: (1) protect the food rewards produced by the plant from food robbers; (2) defend the plant against herbivores and nearby vegetation; and (3) defend the colony against invader ants that may take over the host tree. Accordingly, many of the coevolved traits of *Pseudomyrmex* ants on acacia trees (*Vachellia*, formerly *Acacia*) are behaviors related to defense and aggression For instance, compared to other ants in the same genus, acacia ants are aggressive instead of

shy, diurnal and nocturnal instead of only diurnal, and aggressive against vegetation instead of passive (Janzen 1966).

Even more interesting, acacia ants are closely related to species that are parasitic on acacia trees (Janzen 1975). These species are fascinating because their biology is very similar to that of mutualistic acacia ants, in the sense that they nest on similar tree cavities, and feed on the tree rewards. However, the parasitic ants do not exhibit behavior to protect the tree. A well-resolved phylogeny (Ward 1993; Ward and Downie 2005a; Gómez-Acevedo et al 2010a), and the co-occurrence of mutualistic and parasitic species in the same sites, make acacia ants well suited for comparative studies. Ironically, acacia ants received very little attention during almost four decades after Janzen's comprehensive work on their ecology, evolution and behavior (but see Keeler 1981; Mintzer 1982; Cronin 1998).

In this dissertation, I studied the division of labor among workers, and how it relates to behavioral specialization, brain anatomy and colony size. I also studied behaviors influenced by the association with the tree in mutualistic and parasitic acacia ants. To address those questions, I integrated neuroanatomy, behavioral ecology, field biology, morphology, and comparative methods with the ecological knowledge and natural history background discussed above.

In Chapter 1, I describe a previously unknown behavior of extracting food from other ant-defended acacia trees by the parasitic acacia ants *P. nigropilosus*. I evaluate four possible hypotheses explaining how parasitic ants overcome the usually effective defenses of the mutualistic ants: evasion, chemical crypsis, chemical repellence and activity patterns shifted temporally with respect to the assaulted colonies (Amador-Vargas 2012a). When parasitic ants are stealing, resident ants are evaded by stopping, changing their walking direction or walking faster. Resident and parasitic workers have

similar temporal activity patterns. The combination of speed and evasive behaviors allows parasitic ants to access well-defended acacia trees. Parasitic workers can walk 2.6-fold faster compared with any of the three species of acacia-ants from which they usually steal food. Behavioral assays suggest that *P. nigropilosus* do not have chemical repellence but that chemical crypsis may be involved in the evasion strategy.

In Chapter 2, I tested two hypotheses for the effect of colony size on brain anatomy using the acacia ants *Pseudomyrmex spinicola*: the Task-Specialization hypothesis and the Social Brain hypothesis. To my knowledge, this is the first study to date addressing division of labor and brain anatomy in field colonies and in a plant-mutualistic ant. Tasks-specialization increases with colony size, especially in workers involved in defense. Brain regions of leaf-ants and trunk-ants are differentially affected by colony size, which supports the Task-specialization hypothesis and not the Social Brain hypothesis. My findings therefore suggest that workers specialized in defense may have reduced learning abilities relative to leaf-ants, specifically poorer olfactory learning. In societies with monomorphic workers, brain polymorphism enhanced by group size could be a mechanism by which division of labor is achieved, but these predictions remain to be tested.

As part of the coevolution with the acacia tree, acacia ants evolved aggression against neighboring vegetation that they kill by pruning. In Chapter 3, I study acacia-ant behavior of killing vegetation encroaching on a host tree. I document interspecific differences among acacia ants in the size of the area ("clearing") around the host tree that workers clear from encroaching vegetation. I further test for interspecific variation in pruning behavior, at the level of worker's decision making. Also, to test whether head shape – which affects mandible force – correlate with worker pruning decisions, I examined head morphology of four species of acacia ants and of three close relatives that

are also associated with trees but that are not aggressive against encroaching vegetation. As expected, the species making the largest clearings also attack thickest plant tissue and have broader heads than species that prune less; their heads are almost as wide as the predator species that kills the prey by holding it with the mandibles and stinging. Differences in decision-making among workers, as well as anatomical constrains are important when explaining ecological variation in mutualisms.

Finally, in Chapter 4, I tested whether the mutualism with acacia trees affects the orientation abilities of ants. In contrast with central place foragers, who return to a place to provision young, workers of acacia ants typically do not leave the nest (i.e. their host tree) for foraging, so acacia ants are essentially "in-nest" foragers. In the field, we experimentally disoriented workers trying to return to their host tree after visiting an experimental rotating test apparatus, set up on the ground near the host tree. As expected, the most disoriented workers belong to the mutualistic species that walks the shortest distance away from the host tree (*P. nigrocinctus*). Compared to controls, *P. nigrocinctus* workers on rotated discs: walked more slowly, took longer to return to the tree, more often got off the experimental apparatus by a point other than the entrance point, and deviated from a direct path to the host tree. In contrast, control and disoriented workers of the central-place-foraging ant species differed only in the walking angle with respect to the nest. Our results suggest that the obligatory mutualisms with a tree – where nesting and feeding sites co-occur– facilitated in-nest foraging, which has consequences for cognitive abilities of ant workers, such as spatial orientation.

Chapter 1: Run, Robber, Run: parasitic acacia ants use speed and evasion to steal food from ant-defended trees [1]

INTRODUCTION

Parasites are taxonomically diverse and exploit a variety of hosts, although they all have to overcome the host defences to succeed (Iyengar, 2008). Food stores of social insects are frequent target of parasites specialized on stealing already-collected food (henceforth termed 'robbers'; Perfecto & Vandermeer, 1993; Kronauer, 2004; Breed et al., 2012). Robbers of social insects circumvent the colony defences through various stealth strategies. These include evading guards or workers of the victim colony (LaPierre et al., 2007), reducing the 'chemical apparency' (Jeral et al., 1997; Bauer et al., 2009; Witte et al., 2009) that makes them imperceptible to the resident workers (social insects generally recognize nestmates by recognition compounds accumulating on the integument; Hefetz, 2007), producing chemical repellents that have a deterrent effect on the parasitized workers (Martin et al., 2007) and having different temporal activity patterns compared with the victimized species (Richard et al., 2004; LaPierre et al., 2007).

Ants that are obligate plant associates, unlike ground-nesting ants, not only have to defend their nest against robbers, but also they need to defend the food that is not yet stored. These ants usually nest in specialized plant structures (called domatia), and forage on the food produced by the plant (e.g. nectar, food bodies). The acacia ants (*Pseudomyrmex* spp.) are a classic example of an ant–plant mutualism: the ants nest

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inside the swollen spines of the acacia trees (*Vachelia* spp., syn. = *Acacia)* and feed exclusively on the nectar and solid food (Beltian bodies) produced by the tree (Janzen, 1966). The workers defend their host tree aggressively against potential harm (e.g. by attacking herbivores and by pruning vines that threaten to overgrow the host tree (Suarez et al., 1998; Amador-Vargas, 2008).

The food provided by the acacia also attracts arthropods other than the mutualistic ants (e.g. the spider *Bagheera kiplingi* Salticidae; Meehan et al., 2009). A congener to the ant mutualists, *Pseudomyrmex nigropilosus* Emery (Hymenoptera: Formicidae; referred to as *Pseudomyrmex nigropilosa* in Janzen, 1975), has a parasitic association with the tree. Colonies of *P. nigropilosus* consume the food bodies and nectar produced by the acacia tree and nest exclusively in these trees, although the workers do not defend their host tree against herbivore attack (Janzen, 1975).

In the present study, the behaviour of food extraction from other ant-defended acacia trees by *P. nigropilosus* ants is described for the first time, and the strategies that may allow *P. nigropilosus* ants to overcome or dodge the usually effective defences of the mutualistic ants on their host tree are explored. This research evaluates four adaptations that could allow *P. nigropilosus* workers to enter ant-defended trees and steal food. *Pseudomyrmex nigropilosus* ants (hereafter, parasitic ants) may be able to evade resident workers of the parasitized tree because parasitic ants evade aggressive encounters with the resident ants ('evasion hypothesis'); are chemically cryptic ('crypsis hypothesis'); are repellent to the resident ants ('repellence hypothesis'); or are active at different times than the resident colony on the parasitized tree ('temporal activity patterns hypothesis'). These four hypotheses make different predictions, which are tested in the present study.

MATERIALS AND METHODS

Overview of natural history

In Costa Rican dry forest, acacia trees are associated with one of three species of obligatory mutualistic *Pseudomyrmex* ants: *Pseudomyrmex spinicola* Emery, *Pseudomyrmex flavicornis* F. Smith and *Pseudomyrmex nigrocinctus* Emery. Colonies of the three species defend their tree aggressively against herbivores and other ants, and sometimes have aggregations of workers at the base of the trunk that are highly efficient in deterring intruder ants (Amador-Vargas, 2008). Typically, one tree is inhabited by only one colony of ants with a single queen, although the colony can also expand to neighbouring trees. Surprisingly, workers of *P. nigropilosus* are seen entering neighbouring acacia trees that are inhabited and defended by mutualistic *Pseudomyrmex* ants (Fig. 1.1), and returning to their own host tree carrying Beltian bodies or with their abdomen visibly swollen, indicating that they are full of nectar. This behaviour was performed by workers of several colonies during both the dry and rainy seasons from 2007 to 2009. The parasitic workers avoid encounters with the resident workers on the tree from which they are stealing food, and none of the observed intrusions ends in the parasitic ants being caught or attacked by the resident workers.

Study site

The study was carried out during the rainy season (June to July) at Santa Rosa National Park (10°50' N, 85°37'W), Guanacaste, Costa Rica, a neotropical dry forest where two species of acacia trees occur (*Vachelia collinsii* and *Vachelia cornigera*). Field measurements and experiments were performed in colonies of *Pseudomyrmex spinicola* inhabiting *V. collinsii* trees regularly parasitized by *P. nigropilosus*. Measurements of the other ant species were also obtained in the field on *V. collinsii* trees.

Evasion hypothesis

According to the evasion hypothesis, parasitic ants are predicted to walk faster on a parasitized tree compared with on their own tree, and faster than the resident ants of the parasitized trees. The walking speeds of the parasitic ants and of three species of mutualistic acacia-ants (*P. spinicola*, *P. flavicornis* and *P. nigrocinctus*) that are parasitized regularly by *P. nigropilosus* (S. Amador-Vargas, unpublished observations) were measured. To test the evasion hypothesis, two walking speeds ($cm*s^{-1}$) were obtained for each species: (i) basal speed, referring to the normal walking speed when undisturbed, and (ii) running speed, defined as the speed at which ants walk when disturbed. Basal speed was measured as the distance travelled by ants walking both straight and upwards on their host tree for all species, and on the parasitized tree for the parasitic ants, during 10 frames of video (0.33 s). The running speed for the mutualistic ants was measured after a disturbance of their host tree, whereas the running speed for the parasitic ants was measured after a worker had a close encounter with a resident ant on the assaulted tree (when the two ants were less than 1 cm apart). The running speeds of the parasitic and the mutualistic ants were not measured under the same circumstances because parasitic ants do not run when their tree is disturbed and the mutualistic species do not run away from other ants. However, these measurements allow a comparison of the fastest walking speeds observed in these species. The speed measurements were compared using a factorial analysis of variance (ANOVA), with the two walking situations (basal and running) and the ant species as factors and their interaction term. As another measurement of evasion, parasitic ants were observed for evasive behaviours towards resident ants. Using videotape footage, the reactions of parasitic ants when

confronted with a resident ant or a nestmate that was in their way (not farther than 4 cm or approximately 20 thorax lengths away) were qualitatively measured. The reactions were classified as 'stopped', when the parasitic ant was immobile in two consecutive frames of video, and as 'turned' when, from one frame to the next, the parasitic ant changed the direction at which she was walking. Although the parasitic ants also had the option of continuing walking, they never did so (not even when facing nestmates). All changes in direction were easily identified because the ants turned at least 45◦ from their original direction. The frequencies of each of the behaviours (stopping or turning) towards nestmates and resident ants were analyzed using a chi-square test of independence (Sokal & Rohlf, 1995). Additionally, to determine whether the reaction of the parasitic ant was affected by the distance to the nearest ant in front of her, that distance was also measured in the video frame when the reaction occurred. A binary logistic regression with distance as the continuous variable and 'stopped' and 'turned' as the binary response variable was used to test the association between the variables.

Crypsis and repellence hypotheses

According to the crypsis hypothesis, resident ants were expected to detect more easily by odour the presence of intruder ants from other mutualistic species than from the parasitic species. The repellence hypothesis predicts that resident ants should equally detect the odour of parasitic ants and other mutualistic acacia ants, although they should not be attracted to the parasitic ant but deterred. In the field, behavioural assays were performed to assess simultaneously the crypsis and the repellence hypotheses. Live mutualistic *P. flavicornis* ants and parasitic *P. nigropilosus* ants were caged in cylindrical plastic cages (diameter 5 mm, length 2.5 cm) that were closed and individually numbered. These cages prevented visual contact between the resident and the caged ants

but allowed exchange of odours and contact with the antennae through small spaces. The three cages were tied together (two cages containing a worker of either *P. nigropilosus* or *P. flavicornis*, and an empty control cage). A set of three cages (triplet) was then attached to 19 trees inhabited by *P. spinicola* ants (resident ants), all at the same height. Each cage had its individual number visible and a video recorder captured the reactions of the resident ants to the cages during sets of 2 min separated by intervals of 3 min, giving a total of 10 min of observations. Captive ants were released to their respective colony after the observations were finished, and cages were used only once.

From the videos, the numbers of ants that visited (i.e. that touched the cage with the antennae) and bit each cage (i.e. that pressed the cage with their mandibles) were counted. The observations were recorded blindly by cage number, and the content was assigned to the observations only after all the videos were analyzed. According to the crypsis hypothesis, cages with parasitic ants should receive as many visits as the empty cages but less than cages with the mutualistic ants. According to the repellence hypothesis, cages with the parasitic ant should receive fewer visits than the other two cages. The effect of the cage content (empty, parasitic or mutualistic ant; fixed effect) on the number of ants that visited a cage was statistically tested with Friedman's ANOVA for dependent samples (the colonies) because the number of visiting ants to empty cages was not normally distributed (Shapiro–Wilk test, $W = 0.87$, $P = 0.018$). Wilcoxon matched-paired tests were performed when Friedman's ANOVA was statistically significant.

The proportion of ants that bit was calculated from the number of ants that visited each cage; these proportions are therefore independent of each other. According to the crypsis hypothesis, the proportion of ants that bit cages with parasitic ants should be similar to empty cages but less than cages containing the mutualistic ant. For the

repellence hypothesis, the prediction is that cages with the parasitic ant should be bitten proportionally less than cages with mutualistic ants or empty cages. Because the proportions of biting ants were normally distributed (all Shapiro–Wilk tests, $P > 0.3$), they were compared using a blocked ANOVA of two factors: colony (random, block) and content of the cage (fixed). The position of the cage in the triplet (left, centre or right) has no effect on the number of visiting ants (Friedman's ANOVA, $\chi 2 = 2.08$, d.f. = 2, $P = 0.35$) or in the number of ants that bit (Friedman's ANOVA, $\chi 2 = 3.02$, d.f.= 2, $P = 0.22$); thus, this variable was not considered in the statistical analysis.

Differential temporal activity patterns hypothesis.

According to this hypothesis, the parasitic ants should be active at different times than the ants on the parasitized tree. Therefore, the density of resident workers (workers cm–2) on the tree should be negatively correlated with the frequency of intrusions of the parasitic ants. To test this hypothesis, the density of workers in an area of 10 × 2 cm of the trunk base (the entering place for the parasitic ants) was measured in *P. spinicola* colonies. The number of parasitic ants that entered the tree was recorded in bouts of 10 min. The density of resident ants and the rate of entrance of parasitic ants were measured every 2 h (from 8 to 16 h) in three colonies. The association between density of resident ants (ants per cm2) and the rate of entrance of parasitic ants (ants per minute) was tested using Spearman's correlation.

RESULTS

Evasion hypothesis

The evasion hypothesis predicted *P. nigropilosus* parasitic ants to walk faster than the resident ants, and have a higher turning rate. When walking on their own tree, there

was a significant difference in walking speed of the different species ($F_{3,347} = 68.7$, $P < 0.001$): parasitic ants walked as fast as *P. flavicornis* and *P. nigrocinctus*, whereas *P. spinicola* ants were the slowest (Tukey's post-hoc test $P < 0.05$; Fig. 2.2). The basal speed of parasitic ants on their host tree (5.7 ± 2.9 cm s−1*)* was similar to that on the parasitized tree (5.5 ± 3.7 cm s−1; $t = 0.54$, d.f. = 226, $P = 0.58$). In all species, as expected, the running speed was higher than the basal speed ($F_{1,347} = 246$,$P < 0.001$), although the increment varied between species ($F_{3,347} = 57.12$, $P < 0.001$): parasitic ants had a running speed almost four-fold faster than their basal speed, and 2.6-fold than any of the other acacia-ant species. In the mutualistic species, the running speed was not even three times faster than their basal speed (2.6-fold in *P. spinicola*, 1.5- fold in P. *flavicornis* and 1.4-fold in *P. nigrocintus*; Fig. 1.2). An encounter between a parasite and a resident ant was never observed in the more than 200 incursions recorded of parasitic ants on trees with acacia-mutualistic ants.

The other evasion strategy tested was the reaction of *P. nigropilosus* workers to an ant walking towards them. When facing resident ants, parasitic ants turned more often (35 out of 50) than they stopped (15 out of 50), although not when facing a nestmate (15 stopped and nine turned; $\chi 2 = 7.11$, d. f. = 1, $P = 0.007$). On the parasitized tree, parasitic ants were more likely to stop when they were more than 2 cm away from the other ant, and to turn when they were closer than that (logistic regression: $\chi^2 = 13.3$, d.f. = 1, $P = 0.0002$; Fig. 1.3). On their own tree, the distance to the facing ant did not affect the reaction of the parasitic ants (logistic regression: $\chi^2 = 0.55$, d. f.= 1, $P = 0.46$).

Crypsis and repellence hypotheses

When the three cages were added to the colonies of *P. spinicola*, resident ants are expected to visit and bite more frequently the cages with *P. flavicornis* mutualistic ants,

and less often the empty cages or the cages with parasitic ants. The content of the cage had an effect on the number of visits (Friedman's ANOVA, $\chi^2 = 6.6$, d.f. = 2, $P = 0.03$; Fig. 2.4) but not on the proportion of ants that bit a cage (mean ± SE: empty cages, 0.63 ± 0.05; *P. flavicornis*, 0.65 ± 0.04, *P. nigropilosus*, 0.60 ± 0.05; $F_{2,34} = 0.61$, $P = 0.54$). Resident ants visited cages with *P. flavicornis* mutualistic ants more often than empty cages (Wilcoxon matched pairs test, $Z = 2.05$, $P = 0.04$), although visits to cages containing parasitic ants were not different from visits to empty or mutualistic-ant containing cages (Wilcoxon matched pairs test, $Z = 0.84$, $P = 0.4$ and $Z = 1.44$, $P = 0.14$, respectively).

Differential temporal activity patterns hypothesis

The assessment of the temporal activity patterns of residents and parasites did not agree with the prediction of parasitic ants being active when mutualistic *P. spinicola* ants were less active. Moreover, there was a positive association between the density of resident ants on the trunk and the number of parasites that entered the tree per min (Spearman's $r = 0.72$, $P = 0.008$; Fig. 1.5).

DISCUSSION

The results of the present study support the evasion hypothesis to explain how parasitic acacia-ants (*P. nigropilosus*) enter colonies of the mutualistic acacia-ant *P. spinicola* and steal food. As is predicted by the evasion hypothesis, *P. nigropilosus* ants walk faster than resident ants on the parasitized tree. The speed data suggest that it is not the basal walking speed *per se* that sets the parasitic ants apart from the mutualistic ants but, instead, their ability to increase the speed when they are in a dangerous situation. Ant species with larger body sizes do not necessarily have a faster basal speed or ability to

run: workers of *P. flavicornis* are similar in size to *P. nigropilosus* (thorax length 1.85 ± 0.15 and 1.86 ± 0.28 mm, respectively; mean ± SE; S. Amador-Vargas, unpublished observations), although their running speed is only 1.5-fold faster than their basal speed. The leg length relative to the body size varies among species, and relatively longer legs should allow faster walking (Ritzmann *et al*., 2000; Hurlbert *et al*., 2008). However, the acacia ant species with the longest legs is the mutualistic *P. spinicola* and not the robber *P. nigropilosus* (S. Amador-Vargas, unpublished observations). The running speed of *P. nigropilosus* is approximately 2.6-fold faster than the fastest mutualistic acacia-ants, and it appears to be used by parasitic ants to prevent encounters or to escape when they are chased by the resident ants.

Consistent with the evasion hypothesis, the robbing strategy of *Menemerus* spp. jumping spiders that steal food from loaded ant foragers is similar to the strategy of *P. nigropilosus* ants. *Menemerus* spiders track the ant's movements, take the food from the ant mandibles and rapidly walk away (Jackson *et al*., 2008). Although *P. nigropilosus* ants are extracting food from the tree and not from the ant's mandibles, they also track the movements of resident ants to adjust the walking direction, and accelerate to prevent encounters with the resident ants. This suggests that robber species from different taxa that steal ant-collected or ant-defended food could be using similar behavioural robbing strategies.

Speed is important but not the only component of the evasion strategy of *P. nigropilosus*. Parasitic ants combine speed with other evasive behaviours, such as stopping or changing their direction when facing a resident ant on the parasitized tree. Jumping spiders that also extract Beltian bodies from acacia trees evade resident ants by combining speed with other behaviours, such as jumping off the leaves when hanging from the silk (Meehan *et al*., 2009). Parasitic ants have adapted other behaviours to

extract food from ant-defended acacias. Surprisingly, parasitic ants also react towards nestmates when going up on their own tree. Two parasitic ant nestmates walking towards each other on their host tree rarely interacted with their antennae, which is a regular behaviour seen in the other species. Workers of several species of *Pseudomyrmex* are solitary foragers (Janzen, 1966; Hölldobler & Wilson, 1990), and these data suggest that interactions between workers of *P. nigropilosus* outside the acacia spines rarely occur. Further research could explore the chemical profiles of *P. nigropilosus* ants, and their abilities and mechanisms used to recognize nestmates, if they are not relying on chemical cues.

The chemical repellence hypothesis is not supported by data because *P. spinicola* ants do not avoid cages with *P. nigropilosus* ants. The crypsis hypothesis is partially supported by the number of visits to the cages, although not by the aggression of the ants (biting behaviour). The number of visits to cages with *P. nigropilosus* is intermediate between the other two cages (empty and with *P. flavicornis*). This suggests that, in some colonies, resident ants detect empty cages as much as they detect cages with parasitic ants, although that situation never occurred between empty cages and cages with *P. flavicornis*. Reduced amounts of cuticular compounds could explain why, for some colonies, the ants visit cages with *P. nigropilosus* as often as empty cages. The mutualistic acacia-ants are well known for their aggressive response against anything that contacts their tree (Janzen, 1966; Amador-Vargas, 2008, 2012), which could explain why the resident ants even attack the control cages.

Parasites that steal food without force from social insects usually evade the chemical recognition system of the other species (Breed *et al*., 2012); for example, by reducing the chemical apparency (Jeral *et al*., 1997). The reactions towards caged ants and towards nestmates in their host tree suggest that parasitic *P. nigropilosus* may have

reduced cuticular recognition compounds, as is also true for other parasites (Kroiss *et al*., 2009; Lenoir *et al*., 2001). Reduced chemical apparency and not chemical mimicry also explains why workers from the same *P. nigropilosus* colony are able to parasitize several ant colonies of different species simultaneously (S. Amador-Vargas, unpublished observations). The social parasite *Formicoxenus nitidulus* is also capable of exploiting several species of ants by using a deterrent substance (Martin *et al*., 2007), although this does not appear to be the strategy used by *P. nigropilosus* ants.

There are no indications of differential temporal patterns of activity to account for the ability of *P. nigropilosus* to steal food from other mutualistic acacia ants. The pattern of nectar production by the acacia trees may prevent the shift in the patterns of activity between parasites and mutualists. The tree produces almost no nectar from 07.00 to 09.00 h (2–4 h after sunrise), although a larger amount of nectar is produced from 10.00 to 13.00 h (5 to 3 h after sunrise under LD 12 : 12 h; S. Amador-Vargas, unpublished observations). This appears to coincide with the appearance of more resident ants coming outside of the spines during that time of the day (Raine *et al*., 2002; Clement *et al*., 2008). Adult acacia ants rely heavily on nectar consumption to support their daily energy demands, and nectar is secreted by the extrafloral nectaries primarily during the day. Because nectar is not stored, nectar stealing requires robber ants to be active during the same time as resident ants. On the other hand, the Beltian bodies are available all day long, and parasitic ants could take them at any time, including night, as long as it is before the resident ants have collected them. However, the acacia ants (including the parasites) are diurnal and highly visual, and probably need light to orient themselves (Janzen, 1966). Future work should try assessing the items that are extracted from the assaulted tree by the parasitic ants at different times during the day to understand better

whether parasitic ants are using parts of the tree and resources at different times than the resident ants.

In summary, *P. nigropilosus* ants enter ant-defended trees of acacia using a combination of evasive behaviours, fast speed and probably reduced cuticular compounds (odours). A direct test of the crypsis hypothesis could complement the behavioural observations that suggest a reduced amount of cuticular hydrocarbons. A further exploration of the behavior of parasitic and mutualistic acacia ants could help to understand the evolution of the parasitism, as well as the maintenance of the mutualism. Further research may include comparative phylogenetic studies of cuticular hydrocarbon profiles and quantification of the effect of the parasitism on the fitness of the resident colony for the different species of trees and mutualistic acacia ants.

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Figure 1.1. Parasitic acacia ants *Pseudomyrmex nigropilosus* (white arrows) enter trees defended by the mutualistic acacia ants: (a) *Pseudomyrmex flavicornis* and (b) *Pseudomyrmex spinicola*. The parasites extract (c) Beltian bodies (at tips of leaflets) and (d) nectar secreted at extrafloral nectaries of the acacia trees.

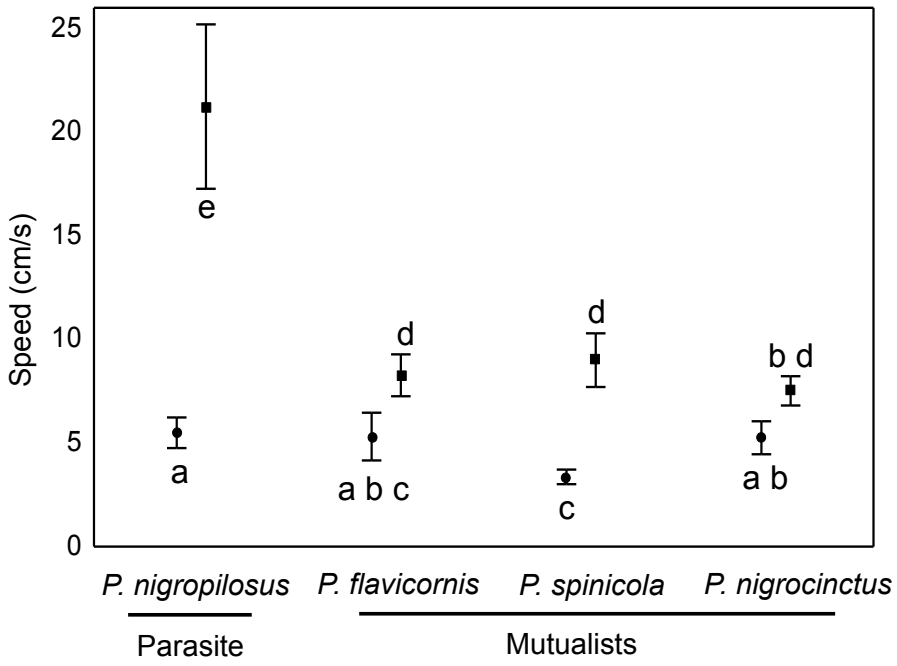

Figure 1.2. Basal speed (circles) and running speed (squares; mean ± SE) of a parasitic acacia ant (*Pseudomyrmex nigropilosus*) and three mutualistic acacia ant species (*Pseudomyrmex flavicornis*, *Pseudomyrmex nigrocinctus* and *Pseudomyrmex spinicola*). The running speed of parasitic *P. nigropilosus* workers was almost four-fold faster than their basal speed, and more than two-fold faster than the running speed of workers of the mutualistic species.

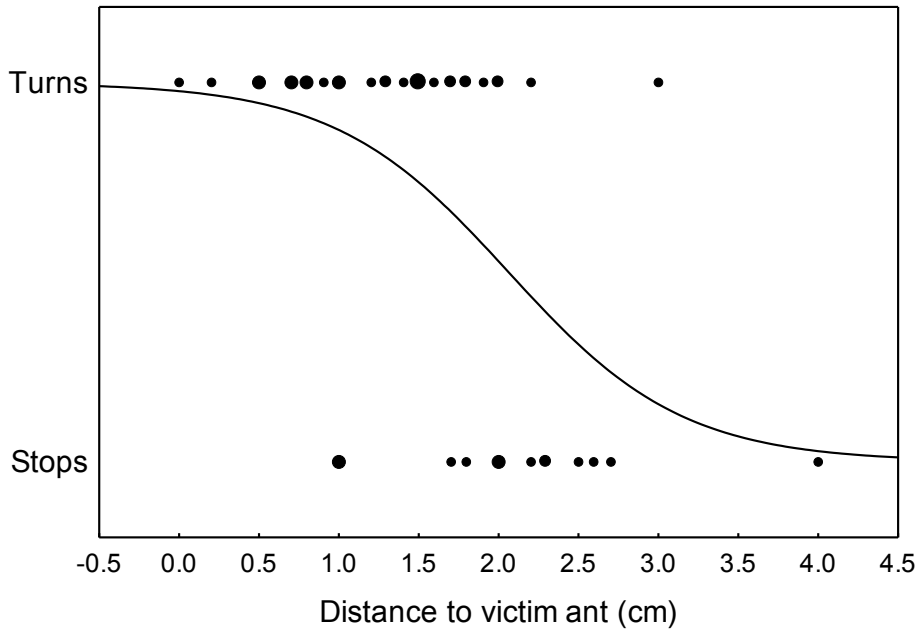

Figure 1. 3. Reactions of parasitic acacia ants *Pseudomyrmex nigropilosus* towards a resident ant (*Pseudomyrmex spinicola*) on the parasitized tree. The circle size represents the number of cases (smallest dots are one observation), and the line is the logistic function of the regression ($\chi 2 = 13.3$, d.f. = 1, $P = 0.0002$). Most parasitic ants turned when the resident ant was less than 2 cm away.

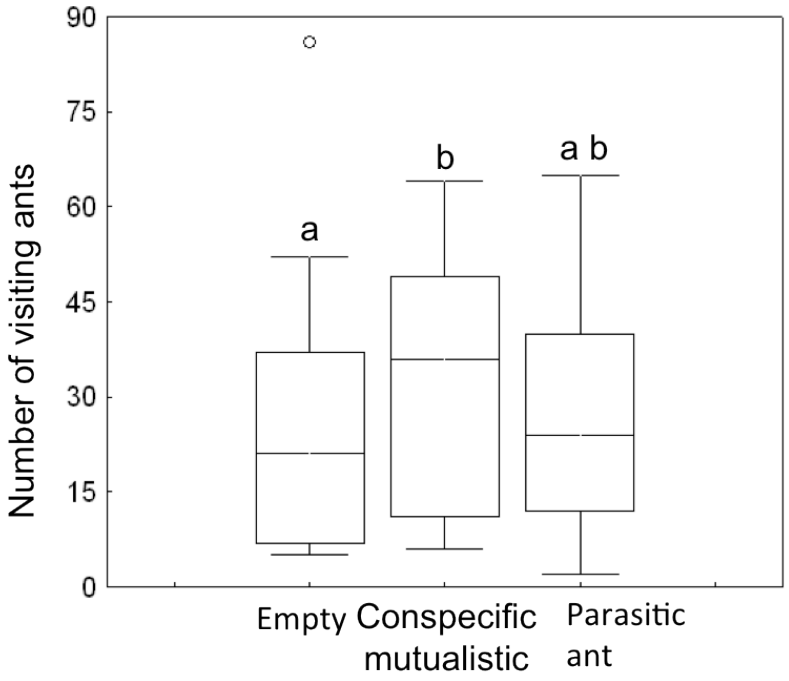

Figure 1. 4. Reactions of parasitic acacia ants *Pseudomyrmex nigropilosus* towards a resident ant (*Pseudomyrmex spinicola*) on the parasitized tree. The circle size represents the number of cases (smallest dots are one observation), and the line is the logistic function of the regression ($\chi 2 = 13.3$, d. f. = 1, $P = 0.0002$). Most parasitic ants turned when the resident ant was less than 2 cm away.

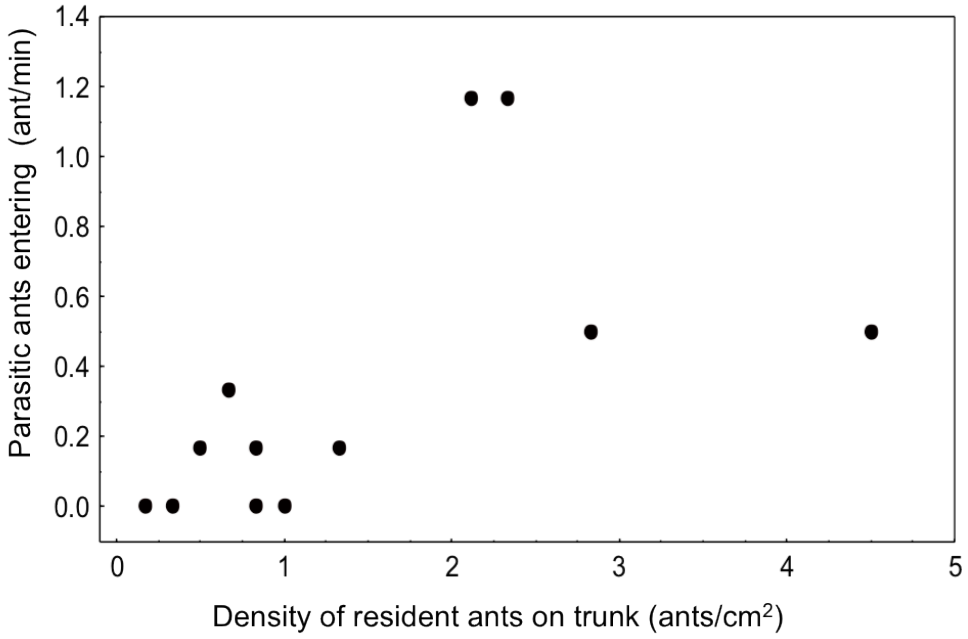

Figure 1. 5. Rate of entering acacia trees of parasitic ants (*Pseudomyrmex nigropilosus*) compared with the density of resident *Pseudomyrmex spinicola* ants on the plant trunk. The activity of the parasitic ants was positively correlated with the density of resident ants on the trunk.

Chapter 2. Specialization and group size: brain and behavioral correlates of colony size in ants lacking morphological castes[2]

INTRODUCTION

Multicellularity and sociality are two of the major evolutionary transitions (Szathmáry and Maynard Smith 1995), and both transitions led to a great diversity in group size (i. e., number of units that form a society or multicellular organism; Bourke 2011). Group size is positively associated with the degree of reproductive and non-reproductive division of labor in both multicellular organisms (e.g. in volvocine algae, Herron and Michod 2008) and social organisms (e.g. ants; Anderson and McShea 2001, Bonner 2004). Individuals in a group can specialize on a set of tasks required for the efficient functioning of the group, which leads to division of labor. While some of the most celebrated examples of morphological differentiation occur among castes of ants, most eusocial societies show division of labor with little morphological differentiation among individuals. In ant species with morphologically similar workers (monomorphic ants), colony organization depends on task partitioning that emerges from body size-independent behavioral specializations of the individuals (Bourke and Franks 1995), rather than from body size-dependent specializations of polymorphic ant species. Typically, when a colony has few individuals, all workers perform essentially the same tasks; but as the colony grows, workers become increasingly more specialized on a subset of tasks. Theory predicts higher task specialization in larger colonies (e.g. Pacala et al

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1996; Bourke 1999; Anderson and McShea 2001, Gautrais et al 2002), but empirical studies to date have generated conflicting support (support: Thomas and Elgar 2003 and Holbrook et al 2011; no support: Dornhaus et al 2009).

Anatomical modifications of the brain and the nervous system undoubtedly relate to behavioral specialization, but the relationships are unclear (Eberhard and Wcislo 2011). Neural tissue is energetically costly, thus the relative size of brain regions is thought to correspond to each region's relative importance for behavioral performance (Laughlin et al 1998; Niven and Laughlin 2008). Workers of social insects performing tasks that require different sensory or cognitive abilities should therefore also have neuroanatomies that reflect their behavioral specialization. For example, in human societies, professions that require efficient spatial orientation (e.g., taxi drivers) have enlarged posterior hippocampi (a brain region involved in spatial memory) compared to those specialized on tasks not requiring efficient spatial orientation (Maguire et al 2000; Maguire et al 2006). Experience performing specific kinds of tasks thus can result in alterations in brain anatomy. The brain also undergoes developmental changes during aging, especially the mushroom bodies (Withers et al 1993; Gronenberg et al 1996; Muscedere and Traniello 2012), but there are also age-independent changes in the brain, which are primarily induced by experience (Gronenberg et al 1996).

Two hypotheses explain how group size could affect brain size and anatomy: the "Task-specialization hypothesis" and the "Social Brain hypothesis". The task-specialization hypothesis, proposes that that as group size increases, task specialization and the relative size of brain regions required to performing those tasks should increase (Gronenberg and Riveros 2009). In contrast, the social brain hypothesis assumes that living in larger social groups imposes greater requirements for cognitive processing, leading to larger brains and especially to a larger relative size of the integration centers in

the brain (Dunbar 1998; Adolphs 2001; Dunbar 2003; Byrne and Bates 2007; Dunbar and Shultz 2007; Pérez-Barbería et al 2007). These two hypotheses also differ in the underlying assumptions: the Social Brain hypothesis assumes that individuals recognize other members of the society as individuals (i.e. using individual-level signals), instead of as members of a group (i.e. using group-level signals), which would be the selective pressure for increased memory and learning as society size increases (Gronenberg and Riveros 2009; Riveros et al 2012). In social insects, the effect of social life on brain size and anatomy has been tested by comparing solitary and social species (Farris and Schulmeister 2011); and facultatively social species in their solitary and social phases (Smith et al 2010). Multispecies comparisons allow testing these hypotheses through a large range of colony sizes, but the results are difficult to interpret because species differ in morphologies and life-history traits. Ants that are obligate plant associates allow an accurate test of these hypotheses, because the colony is limited to the host-plant and can be more accurately quantified in the field than for soil-nesting ant species, and several species have monomophic workers (i.e. all workers have similar external morphology).

We evaluated the Task-specialization hypothesis and the Social Brain hypothesis in the monomorphic acacia ants (*Pseudomyrmex spinicola*; Fig. 2.1). Acacia ants are obligatorily associated with acacia plants (*Vachellia collinsii*, formerly *Acacia*): they nest in hollow spines distributed along branches of the host-tree, forage on the food bodies (called Beltian bodies) and nectar provided by the host-plant on the leaves, defend the tree against herbivores, and kill nearby vegetation competing with the host-plant (Fig. 2.1b; Janzen 1966; Amador-Vargas 2008; Amador-Vargas 2012a). Workers show site-fidelity and behavioral differences according to the site on the host-plant where they work: those located at the base of the tree trunk are more often exposed to ant intruders invading the colony tree by the trunk than to brood or food for the larvae, and show

aggressive responses toward food. In contrast, ants working on the leaves are rarely exposed to intruder ants, but they are frequently in contact with food and brood because they store the collected food bodies inside the spines (Fig. 2.1c; Amador-Vargas 2008; Amador-Vargas 2012b).

In this study, we evaluate the Task-specialization hypothesis and the Social Brain hypothesis by examining the relationship between colony size, task specialization, and brain anatomy. We first tested two assumptions of the Tasks-specialization hypothesis: (1) site fidelity (trunk vs leaves) and working on site-related tasks (foraging vs. defense) increase with colony size; and (2) higher task-specialization is associated with changes in behavior toward stimuli (e.g., food and intruders), resulting in greater behavioral difference between ants specialized in different tasks as colony size increases. Finally, we evaluate the following predictions of the two hypotheses for the effects of colony size on brain anatomy: the Social Brain hypothesis predicts that, as colony size increases, total volume and relative size of the integration centers (called mushroom bodies in insects) should also increase for all workers regardless of task specialization; whereas the Task-Specialization hypothesis predicts increases the relative size of brain regions associated with the task of specialization.

MATERIALS AND METHODS

Study site

This study was carried out at Parque Natural Metropolitano (8° 59' N, 79° 32' W), Panamá City, Panamá, during the rainy season of 2011(July to September). The area is covered by secondary dry forest, including the host-tree *Vachellia collinsii* acacia trees

(formerly *Acacia collinsii*) inhabited by *P. spinicola* ants. Each tree is inhabited by a single colony of ants with one queen.

Task-specialization

Site fidelity in *P. spinicola* is associated with behavioral differences in task-specialization (Amador-Vargas 2012c). Hence, we measure task-specialization as the percentage of workers that were found working on the same task than when they were originally marked on a previous day. To obtain those percentages, we marked (1) workers foraging for Beltian bodies produced at the tip of the leaflets on young leaves (Fig. 2.1c); (2) and workers defending the base of the trunk (Fig. 2.1b), which stand still with the head and antennae directed towards the ground, as described in Amador-Vargas (2008). In 17 colonies living on acacia trees with a range from 5 - 113 in the number of spines (hollow thorns used by the ants as nest cavity), we marked a total of 2688 ants, with an average of 158 ants marked per colony and with approximately half for each task per colony (detailed sample sizes in Table 2.4). We marked ants with a small dot of non-toxic odorless paint (following Amador-Vargas 2008), and we used location-specific colors, that is different colors for workers found on the leaves versus the trunk base (hereafter called leaf-ants and trunk-ants respectively). After 24 hours we revisited the colonies and recorded the number of ants of each type of mark that were found in the two tree locations (trunk base and leaves), by scanning for ants on the trunk base (i.e. from the ground up to the first branch of the host-tree), and then scanning the leaves with Beltian bodies. We only counted ants performing foraging or defense behaviors, and did not count ants that were walking. Counting the same ant twice was unlikely, because trunk ants are usually standing still, and ants collecting Beltian bodies spend some time

detaching the Beltian body from the leaflet and then move to a spine to store the food body. All trees were less than 2 m in height, so all branch tips could be easily observed.

From these worker counts, we calculated measures of foraging and defense specialization as the number of ants with a color-mark found in the same location (trunk, leaves) where they were originally marked, divided by the total number of observed marked ants from that color-mark, then multiplied by 100. This measure is the percentage of marked ants that were found engaged in the same task on two consecutive days (i.e. percentage not switching tasks). To assess the accuracy of these estimates based on two counts, in other four colonies we marked workers in the morning of one day (sample sizes were: 50 leaf-ants and 36 trunk-ants in colony 1; 40 and 30 in colony 2; 50 and 60 in colony 3; 290 and 280 in colony 4), and recounted them three more times: in the afternoon of the same day (~5 hours later), and the mornings of the following two days (after approx. 24 and 48 hours after marking). We calculated the standard deviation among those three estimates of each specialization for each of the four colonies. Across colonies, we found a mean standard deviation of 5% ± 2% for defense specialization, and of 4% ± 2% for foraging specialization. Estimates of task-specialization percentages reported in this study of the 17 studied colonies (based on a single observation 24 hours after marking) therefore have approximately a 5% margin of error.

Colony size

Because colony size is correlated with tree size in obligate plant-associated ants (Fonseca 1993; Fonseca 1999), we estimated colony size from tree diameter and number of occupied spines (spines with entrance hole). Two observers independently counted the number of spines of all trees; if these two counts differed, the count was repeated until both observers agreed. Using the records of marked ants (Table 2.4), we estimated the

number of adult workers working outside the spines with the Petersen method of mark and recapture (Krebs 1999). Estimates of number of outside workers ranged between 14 and 700 ants. Because data did not fit a normal distribution, we used non-parametric Spearman correlation to evaluate the correlations between task-specialization percentages (as defined above) and three proxy-measures of colony size (number of spines on tree, tree diameter, and estimated number of workers outside spines). All Spearman correlations were calculated with the package pspearman (Savicky 2009) for R statistics software (R Core Team 2013).

Behavioral assays

To test whether differences in behavior between leaf- and trunk-ants increased with colony size, we assessed the reactions of marked ants towards two stimuli: food (sample size: 27 ± 11 SD ants per colony, for a total of 117 leaf-ants and 97 trunk-ants from nine colonies) and intruders (sample size: 22 ± 6 SD ants per colony, for a total of 81 leaf-ants and 53 trunk-ants from seven colonies). We tested the reaction towards food by placing a Beltian body still attached to a little fragment of leaf on top of leaves or on spines of the tree trunk and waited for a marked ant to find it, following the methods of Amador-Vargas (2012b). We recorded whether the ant picked up the Beltian body with her mandibles to store it inside a spine (hereafter, "stored"), or whether the ant moved it to the edge of the leave and dropped it to remove it from the tree (henceforth, "discarded"). Because defense and foraging specialization increase with colony size, we expected the probability of discarding food to increase in trunk-ants and to decrease in leaf-ants as colony size increased.

We tested the reaction toward intruders by placing workers of sympatric leaf-cutter ants (*Atta colombica*) on the focal acacia trees. To transfer the intruders with

minimal disturbance, we picked up these *Atta* workers from their foraging trails by inducing them to walk onto a small stick and from there onto a leaf or the trunk of the acacia tree. When a marked acacia ant found the intruder (i.e., touched it with the antenna), we recorded whether she attacked (by biting) or ignored it (i.e., walked away). Because defense and foraging specialization increased with colony size of acacia ants, we expected trunk-ants to be more likely to attack the *Atta* intruders, and leaf-ants more likely to ignore the intruders as colony size increased.

To test whether the probability of performing a behavior (discarding food; attacking intruders) changed with colony size, we used generalized estimating equations (Hardin and Hilbe 2012): the response variables were binary (binomial family, logit link), recoded as 1 for discarding and 0 for storing a food body, and 1 for attacking and 0 for ignoring an intruder; the type of ant was a fixed categorical predictor (trunk-ant was the reference group); the percentage of task-specialization was a continuous predictor, and ant-colony was included as a block or random factor. We performed two separate analyses using percentage of foraging specialization and defense specialization as continuous predictors using the function geeglm of the package geepack in R (Højsgaard et al 2005).

Brain anatomy

To obtain brain measurements of ants from colonies of different size, we collected leaf- and trunk-marked ants from eight colonies, and brought them alive to the laboratory facilities of the Smithsonian Tropical Research Institute (10 minutes from the field site) for histological preparation. Brains of ants were immediately removed from the head capsule in phosphate buffered saline (PBS, pH = 7.4), and preserved in fixative (4% formaldehyde in PBS) overnight at 4°C. Brains were washed twice in buffer and once in

water, for one hour each. We stained brains with 1% OsO_4 in the dark for two hours at 4°C, then for 30 min at room temperature; then washed the brains three times for 30 min with distilled water, then dehydrated brains with 50% ethanol for 10 min, followed by 20 min of 2, 2 -dimethoxypropane. Fixation in plastic resin was preceded by two washes in 100% acetone for 10 min each, followed by rotation for 6 hrs in 50:50 acetone: Spurr's (Electron Microscopy Sciences RT 14300) low viscosity resin, 8 hrs in 10:90 acetone: resin, and 8 hrs in 100% resin. Brains were embedded in Beem® capsules, and cured at 60°C for 18 hours. Most brains were sectioned at 7 μm, and the smallest brains were sectioned at 6 μm to get about the same number of sections per brain. Sections were arranged in order on the microscope slide, and stained with 1% toluidine blue, and cover slides were attached with Permount®. We photographed each section (Fig. 2.2a) using a camera (Leica DFC 320) attached to a light microscope with Köhler illumination (Leica DM LB) at the Microscopy and Imaging Facility of the Institute for Cellular and Molecular Biology at The University of Texas at Austin.

We obtained volumetric measurements of brains from digital 3D reconstructions, using Reconstruct software (Fiala 2005; Fig. 2.2b). We first aligned section photographs and drew the contours of the brain neuropiles (excluding cell bodies) superimposed on the brain images (Fig. 2.2a). By coding section-images prior to measurement, brains were aligned and measured blind with respect to colony identity and type of ant. For each brain, we reconstructed and measured the volume of the whole brain, and the following neuropiles: three regions of the optic lobe (lobula, medulla, lamina), olfactory lobes, the vertical and medial lobes of the mushroom bodies, and the lip and collar of the medial and lateral calyces (following Gronenberg 2008). The basal ring was indistinguishable from the collar. All volumetric measures were relativized to the total brain volume, which included the sub-esophageal ganglion. Neuropile volumes are a proxy for the density or

mass of synaptic circuits or the number of axonal and dendritic connections (Strausfeld 2012). We also performed analyses of those measurements relativized by "brain volume remainder", i.e. the total brain volume excluding the brain regions of interest, that is: total volume minus volume of optic lobes, antennal lobes and mushroom bodies. Because results of analysis relativizing by brain volume remainder were congruent with results relativizing by total brain volume we only present the latter results.

To assess the effect of colony size and task-specialization on brain morphology, we first summarized colony size-related traits in a single variable called "colony size-related traits" which is the first factor of a principal component analysis (PCA) including: (a) defense specialization; (b) foraging specialization; (c) number of outside workers; and (d) number of tree spines. This first factor of the PCA explained 56% of the variation between colonies (Table 2.5). We multiplied values of the first factor by minus 1, such that larger numbers indicate larger colonies on larger trees.

We used generalized linear models to test for homogeneity of slopes for a regression between the colony size-related traits (factor 1 of the PCA described above) and the relative brain-region volume between trunk- and leaf-marked ants. The model included the relative volume of a particular brain region as the response variable, and we tested the interaction between type of ant (leaf- and trunk-ants) as a categorical fixed factor and colony size as a continuous factor. A significant interaction in this model means that the continuous variable differently affects the two groups of ants (i.e. that the slopes differ). To correct for multiple comparisons, we used false discovery rates (Storey et al 2004) to calculate a cut-off q-value where the probability of finding a false positive among our significant results was less than one, specifying the bootstrap method in the q-value function of the package "qvalue" for R (Dabney and Storey 2004) . We also report partial omega squared (ω_p^2) with 95% confidence intervals as a measure of effect size,

which were calculated with bootstrapping (1000 repetitions) using the package "boot" for R (Canty and Ripley 2014). We used omega-squared estimates because they are less biased than eta squared estimates, although usually yielding lower effect sizes (Meyers et al 2006).

To evaluate how colony size traits correlated with another non-neural morphological trait, we measured the head size area of trunk- (n = 51) and leaf-ants (n = 43). We took pictures of the ventral view of heads from which we dissected the brains, using a camera (Leica DFC240) attached to a stereoscope (Leica MZ16). In the calibrated image, we measured the head area as the contour of the head excluding the eyes and the mouthparts, using ImageJ (Schneider et al 2012); Fig. 2.2c). We tested for homogeneity of slopes of trunk- and leaf-ants in the correlation between colony size-related traits and head area, as explained above for brain volume.

RESULTS

Site fidelity and colony size

Our results support the assumption of the task-specialization hypothesis that behavioral specialization increases with colony size. The proportion of trunk-ants found working again on the trunk was strongly correlated with the estimators of colony size: number of workers outside spines (Fig. 2.3a), spines on the tree (Fig. 2.3b), and tree diameter (Fig. 2.3c). In contrast, the proportion of leaf-ants found working again on the leaves did not correlate with the estimators of colony size (number of spines, Fig. 2.3d; estimated number of workers, Fig. 2.3e; and trunk diameter, Fig. 2.3f). We also analyzed the dataset excluding the largest colony, because we had a gap in the size range of sampled trees (between 60 and 113 spines; Fig. 2.3a, d); in that case, foraging

specialization positively correlates with number of spines (Spearman r = 0.62, p = 0.01) and number of workers (Spearman r = 0.49, p = 0.051), but not with tree diameter (Spearman r = 0.40, p = 0.11), and trunk specialization still is correlated with number of spines (Spearman r = 0.69, p = 0.003), number of workers (Spearman r = 0.58, p = 0.01), and with tree diameter (Spearman r = 0.57, p = 0.02). The proportion of trunk-ants re-sighted to work on the trunk did not correlate with the proportion of leaf-ants re-sighted to work on the leaves; defense specialization in a colony therefore did not correlate with foraging specialization (Fig. 2.7; Spearman rank-correlation r = 0.38, p = 0.12), but the correlation is marginally significant when excluding the largest colony (Spearman rank-correlation r = 0.43, p = 0.08).

Behavioral assays

We did not find evidence that colony size enhanced the dissimilarity between trunk- and leaf-ants in the reactions towards foraging-related or defense-related items; that is, the presence of more workers specialized in defense or in foraging did not cause them to behave more differently towards stimuli. Specifically, when we tested for worker's reaction toward a Beltian body offered experimentally, the likelihood for a worker to discard this food-item decreased as foraging specialization increased, but this decrement depended on the type of ant. Specifically, for trunk-ants a one percent increase in foraging specialization decreased the odds of discarding by 4% (exponentiated β_2 in Table 2.1, Fig. 2.4), whereas for leaf-ants the odds of discarding increased by 0.03% ($\beta_2 + \beta_3$ in Table 2.1; Fig. 2.4). Defense specialization did not have an effect on the probability of discarding food (Table 2.6, Fig. 2.8). In the intruders test (presenting an *Atta* worker), the odds of attacking were 2% lower for leaf-ants than for trunk-ants (Table 2.7), but

these odds were not affected by the percentage of foraging or defense specialization (Tables 2.7 and 2.8, respectively).

Brain anatomy

Overall, brains of leaf and trunk-ants did not differ in total volume (Table 2.2; $F_{1,59} = 0.98$, $p = 0.33$; ω^2=0.0003, CI: 0 - 0.10). Optic lobes and mushroom bodies comprised similar proportions of the total brain volume (about 15%) and olfactory lobes comprised 6% of the brain volume (Table 2.2). Colony size-related traits (first factor of a PCA combining host tree traits and mark-recapture estimates, see methods) had a marginally significant effect on total brain volume that was different between leaf- and trunk-ants (Fig. 2.5a; interaction term of Ant type * Factor 1-Colony traits, $F_{1, 59} = 3.3$, $p = 0.07$): total brain volume of trunk-ants increased with colony size-related traits ($r^2 = 0.35$, C.I = 0.018 – 0.61; $p = 0.04$), whereas total brain volume of leaf-ants was not affected by size-related traits ($r^2 = -0.08$, C.I = -0.44 – 0.28; $p = 0.65$). In contrast, head area showed a positive correlation with colony size (Fig. 2.5b; $F_{1, 93} = 16.05$, $p < 0.0001$, ω_p^2= 0.14, CI: 0.04 - 0.25), regardless of the type of ant (interaction of colony size and ant type, $F_{1, 93} = 2.23$, $p = 0.14$; ω_p^2= 0.010, CI: 0 - 0.064).

Three brain regions within the calyces of mushroom bodies were differently correlated to traits related to colony size for trunk and leaf-ants. The lip of the medial and lateral calyxes (Fig. 2.6a, b) and the collar of the lateral calyx (Fig. 2.6c) were relatively smaller in trunk-ants living in larger colonies (more tree spines and workers, higher defense specialization), than in trunk-ants defending smaller colonies. Conversely, for leaf-ants, those same regions increased in relative size as colony size increased (Fig. 2.6, Table 2.3). The interaction between colony size-related traits and type of ant explained

about 18% of the variation in lateral lip volume, and 8.5% and 5.6% of the variation in medial and lateral collar respectively.

Correlations of sensory neuropiles in the brain with colony size-related traits were not affected by task specialization. The relative volume of some regions within the optic lobes slightly increased with colony size: the lamina and medulla tended to be larger for workers living in larger colonies, regardless of task, although colony size explained, respectively, only 4% and 3% of the variation, and the confidence intervals for effect size include zero (lamina: $F_{1, 59} = 3.56$, $p = 0.06$, $\omega_p^2 = 0.04$, CI: 0 - 0.19; and medulla: $F_{1,59} = 2.90$, $p = 0.09$, $\omega_p^2 = 0.03$, CI: 0 - 0.17; Fig. 2.9). Mushroom body lobes and the olfactory lobes were not statistically correlated with colony size-related traits neither did they differ between leaf-ants and trunk-ants (Table 2.3, Fig. 2.10).

DISCUSSION

Of the two hypotheses contrasted in our study testing the effect of colony size on worker behavior and brain anatomy in the acacia ant *Pseudomyrmex spinicola* (Task-Specialization Hypothesis and Social Brain Hypothesis), our observations support the hypothesis that task-specialization increases with colony size because larger colonies exhibited more specialized workers engaged in defense and foraging behaviors (Fig. 3). This higher specialization in defense and foraging in larger colonies was correlated with task-dependent anatomical changes in the relative volume of brain regions, specifically in subregions within the integrations centers (mushroom bodies), which likewise supports the Task-Specialization hypothesis at the neurological level.

Behavioral Tests of Task-Specialization and Social Brain Hypotheses: Behavioral observations confirmed one of the two assumptions of the Task-Specialization

hypothesis: that task-specialization increases with colony size. Our measurements of task-specialization actually refer to site fidelity, because we are observing whether ants return to the same place to perform site-related tasks. Fidelity to a site in the colony may facilitate task-specialization (Wilson 1971; Sendova-Franks and Franks 1993); hence, when acacia ants return to the same location to work after spending the night inside the spines, they are repeatedly exposed to the same stimuli and less frequently to other stimuli, which could then induce the observed behavioral and neuroanatomical differences. In large colonies, a higher fidelity to a particular tree location and exposure to task-related stimuli correlated with the behavior of the workers: trunk ants are more likely to discard food, and are more prone to attack intruders than leaf ants (Amador-Vargas 2012c). Hence, our observations support the first assumption of the task-specialization hypothesis.

A second assumption of the Task-Specialization hypothesis is that colony size enhances the behavioral differentiation among workers. Trunk-ants were more likely to discard food, but contrary to the expectation of task-specialization hypothesis, the likelihood of discarding food was not affected by the degree of defense specialization but by the degree of foraging specialization. In other words, trunk-ants were less likely to discard food when there were fewer leaf-ants in the trunk. The growth of ant colonies that have obligatory mutualism with plants is largely limited by food provided by the host tree (Fonseca 1993), and large colonies of acacia ants are often seen collecting food bodies from nearby acacia saplings (Amador-Vargas 2012b). A possible explanation for the observed decrease in the likelihood of discarding food as foraging specialization increased could be that large colonies suffer stronger selection acting against food-discarding behaviors caused by high food demands, although we lack studies that assess how food production and colony demand change with colony size. Likewise, in the

intruders assay leaf-ants were less prone to attack than trunk-ants but this behavioral difference was not affected by colony size as we expected. Colonies may be able to tolerate leaf-ants that ignore some intruders, but trunk-ants must be least tolerant because the fitness of the entire colony largely depends on how trunk-ants guard and defend the acacia tree (Janzen 1966). Therefore, although we tested only two behaviors that could be affected by colony size, our observations fail to support the assumption of the Task-Specialization Hypothesis of a greater behavioral difference between workers in larger colonies.

Brain-Anatomical Tests of Task-Specialization and Social Brain Hypotheses: Worker subcaste-differences in brain anatomy agree with predictions of the Task-Specialization Hypothesis, and are inconsistent with the Social Brain Hypothesis. The mushroom bodies are crucial for the Social Brain hypothesis as they are involved in multisensory integration, memory and learning in the insect brain, and this hypothesis predicted an increase on the relative size of the mushroom bodies with group size for all society members, regardless of task-specialization (Gronenberg 2008; Gronenberg and Riveros 2009; Lihoreau et al 2012). In contrast to this prediction, we found a tasks-dependent effect of colony size and specialization on the relative size of regions within the mushroom bodies calyces, which is consistent with the Task-Specialization hypothesis and contradict the Social Brain hypothesis.

A decrease in the relative size of mushroom bodies with defense specialization was previously unknown for monomorphic workers. Specifically, we observed a decrease in the regions for integration of olfactory (lips) and visual (collar) input. Only one other study with wasps documents the effects of aggressive behavior on the volume of brain regions, and reports the opposite effect of what we found: an increase in the size of mushroom bodies calyces and in the ratio of lips to Kenyon cell bodies (Molina and

O'Donnell 2007). Studies of other ant species with workers specialized in defense (e.g., guards within the major caste of workers in *Oecophylla*, Holldöbler 1983; subordinates in *Dinoponera*, (Asher et al 2013)) may be useful in understanding whether this is a general trend among social insects. In contrast, the observed increase in those same mushroom body regions for foragers is congruent with findings in other social insects such as *Camponotus* ants and honeybees, where the experience of foraging increases the relative size of mushroom bodies (Gronenberg et al 1996; Farris et al 2001).

What are the behavioral implications of relatively larger or smaller lips or collars in the mushroom bodies? Mushroom bodies in general are involved in multimodal sensory processing (Heisenberg 1998; Strausfeld et al 1998; Gronenberg 2008), context generalization (Liu et al 1999), problem solving and decision-making regarding choices (Tang and Guo 2001). Specifically, the mushroom bodies calyxes (containing the lips and collars) receive sensory input, whereas the lobes are in general regarded as output areas. Within the calyces, the lips are the regions where axons from projection neurons of the olfactory lobe synapse with dendrites of Kenyon cells, while the collars have direct visual input from the medulla and the lobula of the optic lobe (Gronenberg 2001). The observed patterns therefore suggest for *P. spinicola* that workers from large colonies specialized in defense may have reduced olfaction-related processes (e.g., learning, decision-making). Our anatomical brain measurements suggest for future behavioral studies comparing not only reactions towards stimuli used in our study, but also learning abilities among workers specialized in different tasks or among workers from colonies of different sizes.

The reported correlation of colony size and brain anatomy also have important implications for the symmetry and function of the mushroom bodies calyces: the observed changes in relative size of lip and collar in foragers and workers involved in defense were stronger on the lateral than on the medial calyx. Studies on axon projections

regularly document mirrored projections to both calyces, and therefore they are expected, and often assumed to be symmetrical (Gronenberg et al 1996; Strausfeld 2012; Nishikawa et al 2012; Giraldo et al 2013). However, Riveros and Gronenberg (2010) also documented stronger effects of foraging experience on one calyx of bumblebees (i.e., foraging experience affected the medial but not the lateral calyx); and based on morphometric measurements across species, Jaffe and Perez (1989) proposed that asymmetry between calyxes increased with colony size and worker polymorphism. These results underline for future studies the need to distinguish between calyxes when studying brain anatomy, and also to explore the respective functional differences.

We could not find evidence of an overall increase in absolute brain size intra-specifically in *P. spinicola*, whereas some comparative studies have shown that workers from species with larger colonies have larger brains (Wehner et al 2007; Muscedere and Traniello 2012). Instead, we found a small task-dependent effect of colony size on absolute brain volume (interaction between colony size and type of ant explains 3.4% of the variation), whereas head size increased with colony size for both leaf- and trunk- ants. Hence, in larger colonies workers were overall larger but had relatively smaller brains, which agrees with predictions of the Task-Specialization Hypothesis, but also with the general trend of larger animals having relatively smaller brains (Eberhard and Wcislo 2011). This result implies that other structures inside the head capsule (e.g., glands, muscles, infrabuccal pocket) may be relatively larger in workers from larger colonies, which also occurs in the larger castes of polymorphic ants (Gronenberg et al 1996; Muscedere and Traniello 2012).

One potential shortcoming of our study is that we did not know the workers' age. Working with colonies in the field makes it difficult to track the activity and age of each individual, which prevented us to directly assess the effect of age on neuroanatomy. If

age is responsible for the observed plasticity, then age differences should also be greater in larger colonies, which is currently unknown. However, even if ants working on one task were older than ants working on a different task, our conclusions are still valid because we are documenting how colony size affects the behavioral and neuroanatomical differentiation among workers (which could be mediated by age). Controlling the effect of age for workers in field colonies is one of the challenges we have to better understand how tasks-specialization and colony size can affect the worker behavior and brain anatomy.

Conclusions.

The Social Brain hypothesis and the Task-Specialization hypothesis propose contrasting effects of social life and group size on neural tissue (Gronenberg 2008; Gronenberg and Riveros 2009). We provide empirical evidence to test both hypotheses in the same species of acacia ants, without the confounding effects of the worker's external morphology or natural history differences complicating studies that compare different species. Our study on the acacia ant *P. spinicola* shows that, indeed, workers exhibit greater task-specialization as group size increases especially on defense tasks, which confirms the main assumption of the task-specialization hypothesis. In addition, brain anatomy agrees with predictions of the Task-Specialization hypothesis, as we found task-dependent effects of colony size on brain regions. Specifically, group size and task-specialization correlated with relative volume of integration regions (i.e., mushroom bodies) but not with the sensory regions within the brain. We encourage studies on other structures inside the head capsule for understanding the evolution and plasticity of relative brain size and how it is affected by task-specialization. Processes underlying

learning and memory should be the focus of future studies on behavioral effects of society size.

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FIGURES

Figure 2.1. Monomorphic workers of acacia ants (*Pseudomyrmex spinicola*) (a) nest inside the swollen spines (*s*) of acacia trees, and feed on the nectar produced in extrafloral nectaries (*n*), and on Beltian bodies (*bb*) produced at the tip of the leaf folioles. **(b)** Workers specialized in defense of the trunk base of the acacia tree, which is the access to the tree for other ants. The worker on the trunk (trunk-ant) is showing the typical guarding posture: standing still with the head directed downwards and it is holding with the mandibles an intruder *Crematogaster brevispinosa* ant. **(c)** A worker specialized in foraging on the leaves (leaf-ant) is harvesting a Beltian body to feed the brood; this ant is marked with a green dot on the abdomen to identify it as a leaf-ant.

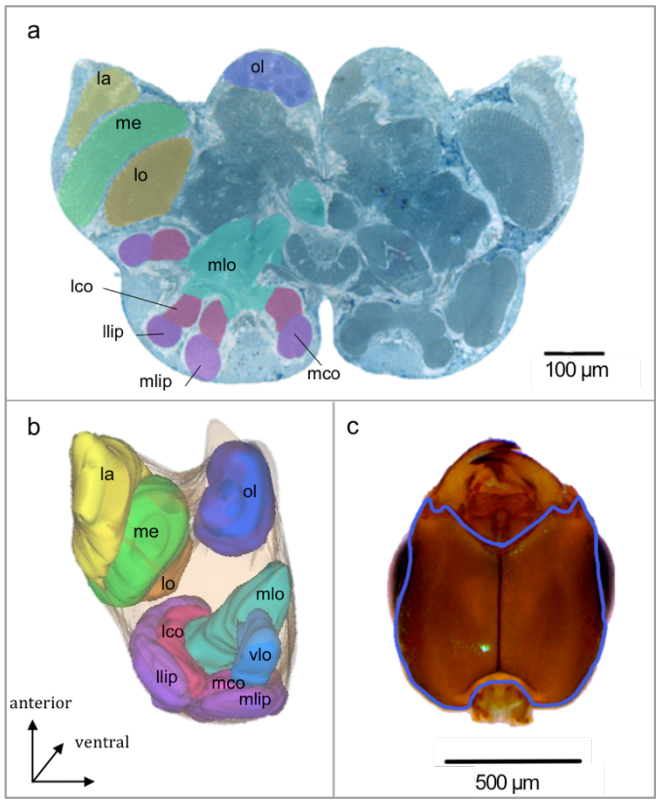

Figure 2. 2. Acacia ant (*Pseudomyrmex spinicola*) transverse brain sections and ventral view of head: (a) Brain histological section highlighting some of the measured neuropiles including: the lamina (*la*), lobula (*lo*) and medulla (*me*) of the optic lobe; and the olfactory lobe (*ol*). In the mushroom bodies, we measured the lip and collar of the lateral (*llip* and *lco*) and medial calyxes (*mlip* and *mco*); the vertical lobe (not visible in this section) and medial lobe (*mlo*). (**b**) Neuropile dimensions on brain sections were used to generate 3D reconstructions of the brain regions from which we obtained the volumetric measurements. Colors correspond to the distinct neuropiles shown in the section, and the vertical lobe not visible in the 2D section is shown here (*vlo*). (**c**) Ventral view of the head showing the contour drawn to calculate the head area, excluding eyes and mouthparts.

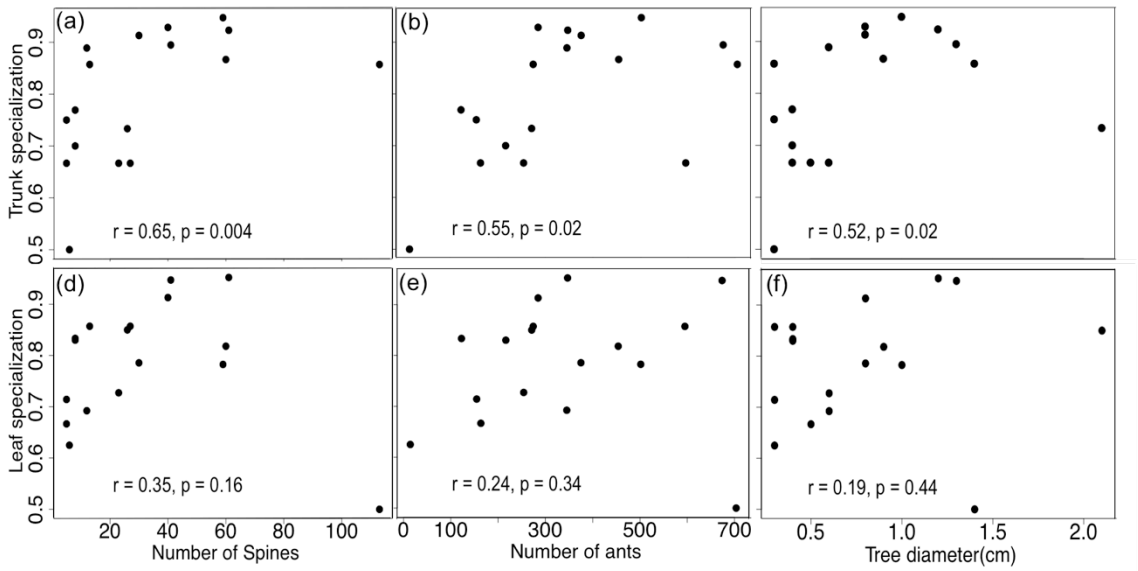

Figure 2.3. Proportion of trunk-ants or leaf- ants that were re-sighted working on the same tasks than the previous day, in relation to three estimators of colony size. Re-observation of an ant performing the same task (i.e., absence of task switching) is a measure of behavioral specialization. The specialization of trunk-ants increased with (**a**) the number of spines on the tree, (**b**) with the estimated number of outside workers, and (**c**) with tree diameter. Leaf-ants were not more specialized in trees with (**d**) more spines, or in colonies with (**e**) more workers or of (**f**) greater diameter. Correlations of leaf specialization with spines and number of workers are statistically significant when excluding the largest colony (see text).

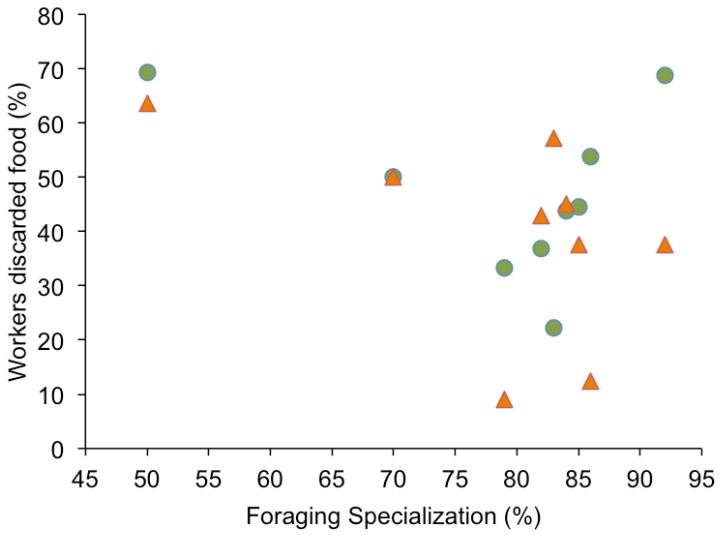

Figure 2.4. Percentage of trunk-ants (orange circles) or leaf-ants (green diamonds) that discarded offered Beltian bodies instead of storing them inside the swollen spines of the acacia tree where they nest, according to the percentage of workers specialized in foraging in the colony. Trunk ants were less likely to discard food as colony size increased, while the odds of discarding almost did not change for leaf-ants (Table 2.1).

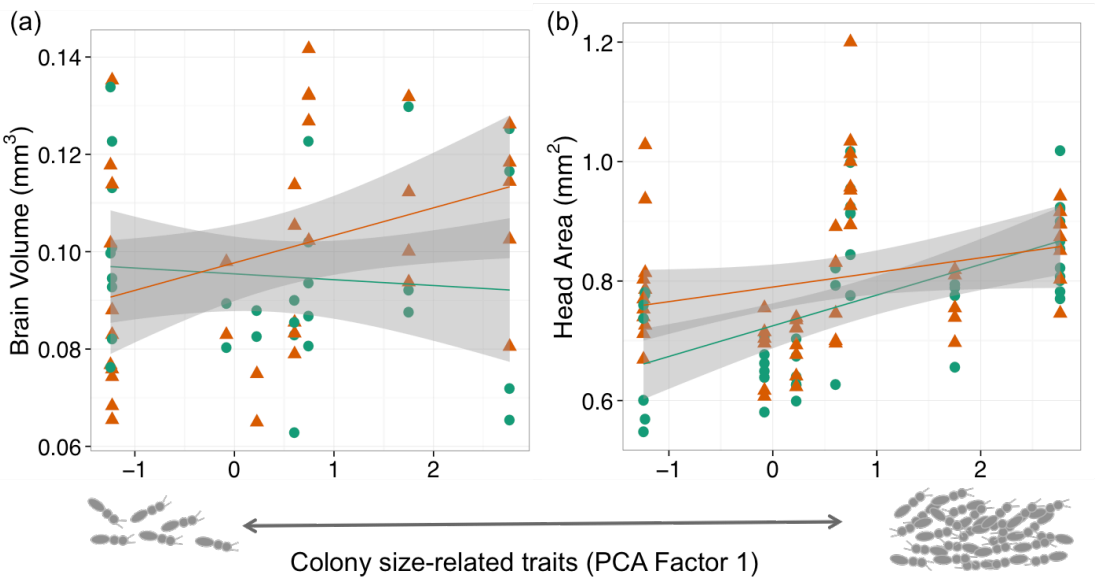

Figure 2.5. Absolute (a) brain volume or (b) head area of acacia ants performing tasks at the trunk (triangles) or foraging at the leaves (circles) of the host tree, as a function of colony size-related traits (larger colonies inhabiting trees with more spines and with higher task-specialization percentages appear towards the right of the X-axis). Gray shadows represent 95% confidence intervals for the linear fit. As colony size and task-specialization increases, absolute volume size tends to increase faster for trunk- than for leaf-ants, while head area increases equally for both types of ants.

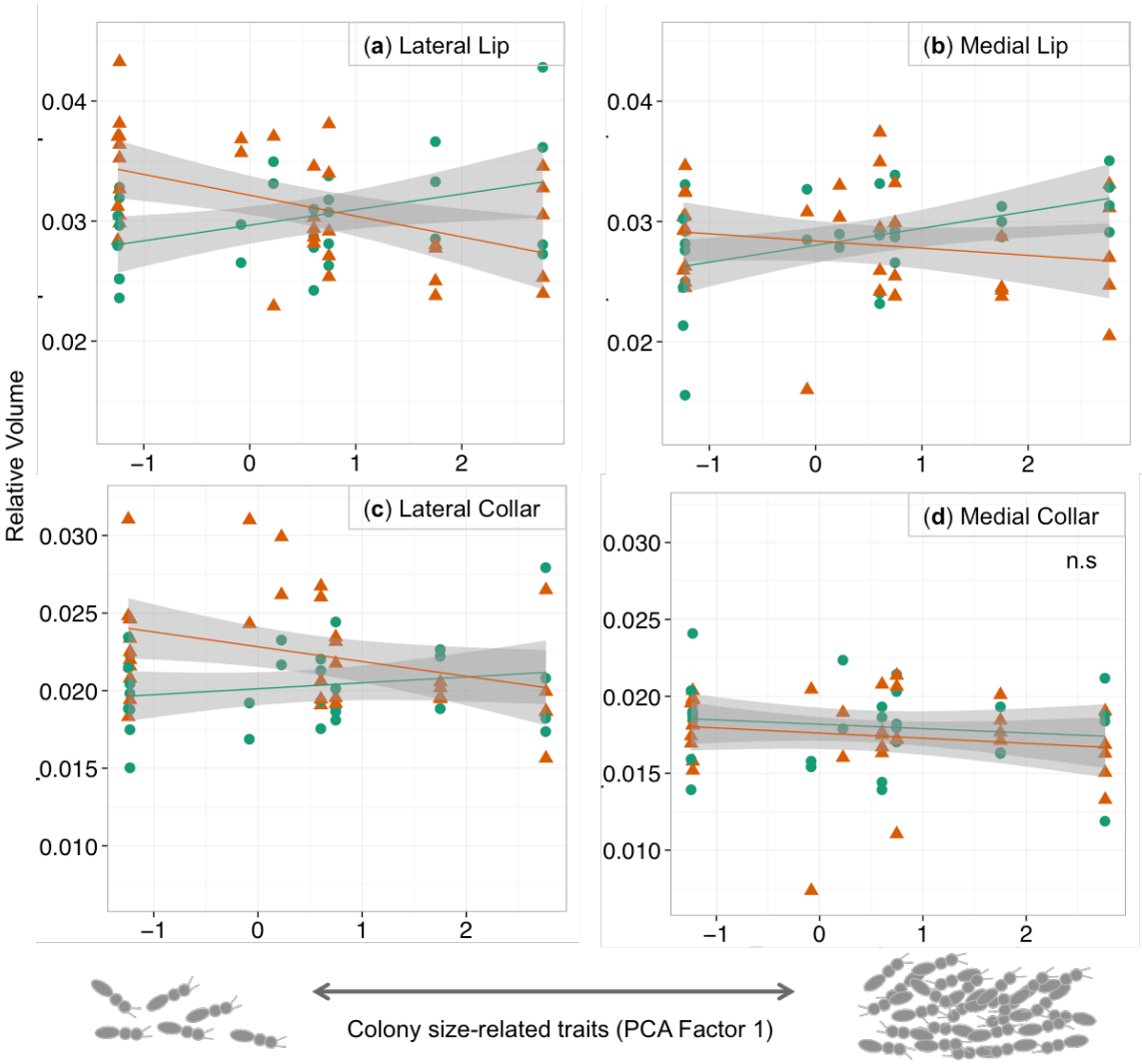

Figure 2.6. Relative volume of mushroom bodies calyces regions, for trunk (orange triangles) and leaf-ants (green circles) according to the colony size traits (larger colonies living in trees with more spines and with higher task-specialization are at the right of the X-axis). Trunk-ants in smaller colonies have relatively larger volume of (a) lateral lip, (b) medial lip and (c) lateral collar than when living in larger societies. Conversely, those same brain regions are relatively larger in leaf ants living in larger societies. The medial collar (d) did not change with colony size traits for any of the two types of ants.

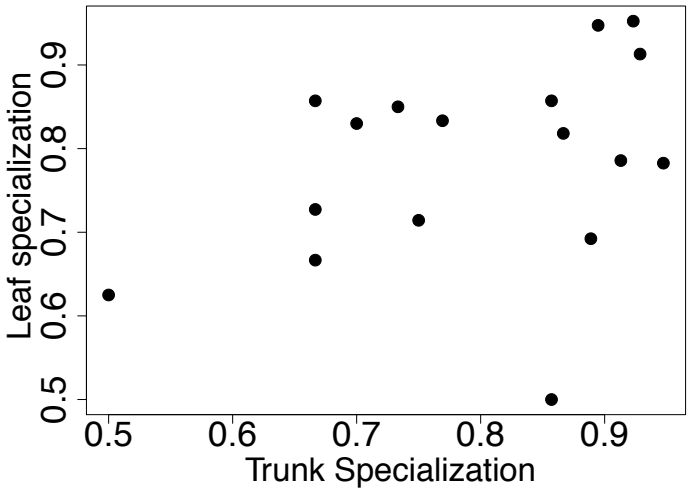

Figure 2.7. The proportion of ants specialized in foraging tasks (i.e., workers that were marked and re-sighted foraging on the acacia tree leaves) **did not correlate with the specialization on defensive tasks** (i.e., returning to work at the trunk base of the host tree). Each dot represents a colony of ants inhabiting one acacia tree.

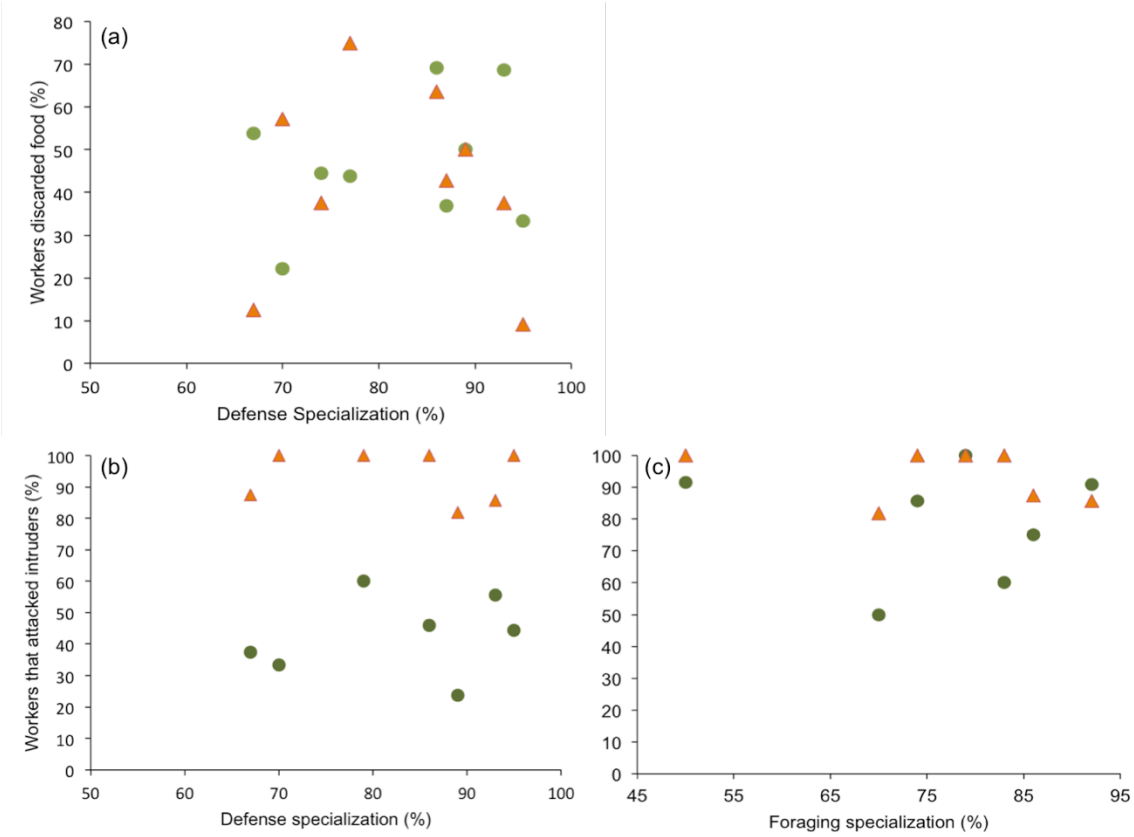

Figure 2.8. Percentage of trunk-marked (orange circles) or leaf-marked (green romboids) acacia ants that (**a**) discarded offered Beltian bodies instead of storing them inside the swollen spines of the acacia tree where they nest according to the percentage of workers specialized in defense (i.e. percentage of trunk-marked ants that returned to defense-related tasks after a day, see Methods) in the colony; or that attacked intruders instead of ignoring them according to (**b**) defense or (**c**) foraging specialization in the colony (i.e. percentage of leaf-marked ants that returned to foraging-related tasks after a day, see Methods)

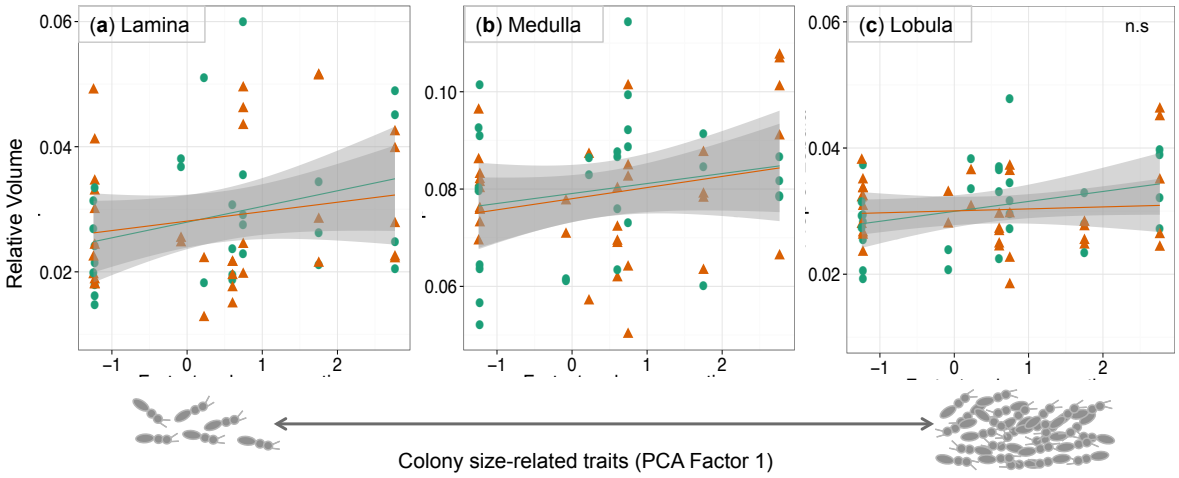

Figure 2.9. Relative volume of the subregions comprising the optic lobes. The lamina and medulla or both trunk-ants (triangles) and leaf-ants (circles) increased in relative size with colony size-related traits. Shaded areas denote 95% confidence intervals.

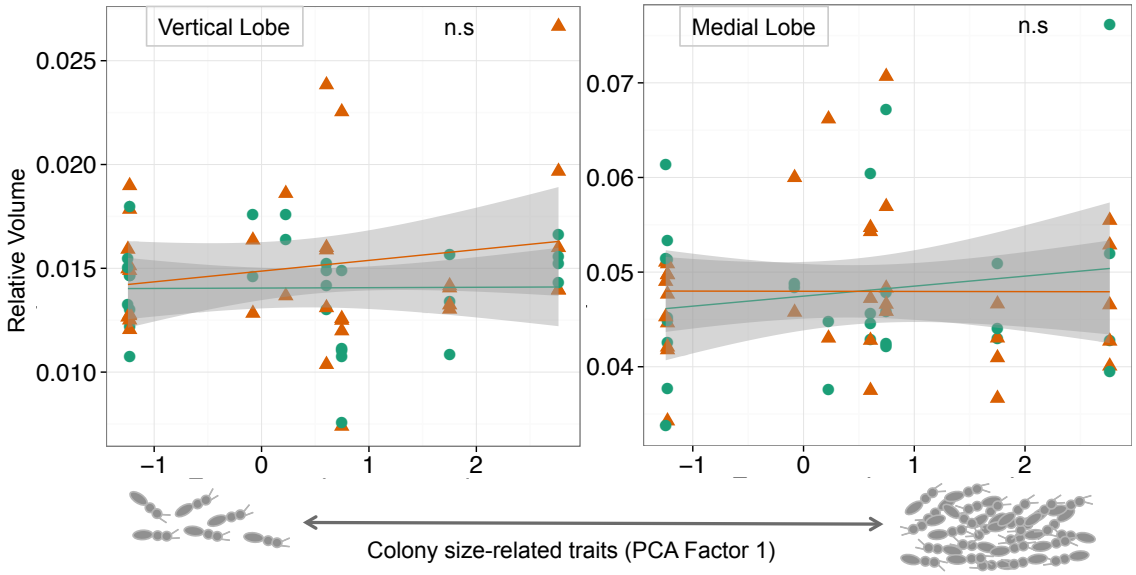

Figure 2.10. The relative volume of the mushroom body lobes was not affected by colony size-related traits. Brains of trunk-ants (guarding and defending) are shown in triangles and brains of leaf-ants (foraging) are represented by circles.

TABLES

Factor	Estimate (log-odds)	SE	Wald	P-value
β_0 Intercept	2.97	0.43	45.87	**<0.0001**
β_1 Ant type (trunk-ants are reference, i.e. when leaf-ants = 0)	-3.04	1.66	3.33	*0.07*
β_2 % Foraging specialization	-0.0425	0.007	31.8	<0.0001
β_3 Ant type* % Foraging specialization	0.0428	0.02	3.53	***0.060***

Table 2.1. Effect of foraging specialization on the log odds of discarding food (log odds of discarding food = β_0 intercept + β_1 Ant type (leaf-ants =1) + β_2 % foraging specialization + β_3 Ant type * % foraging specialization), estimated using a generalized estimating equation. For trunk-ants, the odds ratio of discarding food with a unit increase in foraging specialization is exp (β_2) = 0.96, while for leaf-ants it is exp ($\beta_2 + \beta_3$) = 1.0003. Significant estimates are bolded and marginally significant are in italics.

Brain region	Trunk-ants	Leaf-ants
Total brain volume (mm^3)	0.10 ± 0.023	0.095± 0.019
Optic Lobes %	0.139 ± 0.02	0.137± 0.02
Olfactory Lobes %	0.064 ± 0.015	0.068 ± 0.013
Mushroom bodies, %	0.16 ± 0.017	0.15 ± 0.017

Table 2.2. Brain measures of trunk-ants and leaf-ants inhabiting acacia trees (mean, sd).

Brain region	$F_{1,59}$	P-value	Effect size ω_p^2
Optic Lobes			
Lamina	0.23	0.63	
Medulla	0.008	0.92	
Lobula	1.20	0.28	0.003 (0-0.11)
Olfactory lobes	0.02	0.89	
Mushroom bodies			
Calyces			
Medial Lip	**6.76**	**0.01**	**0.085 (0-0.25)**
Medial Collar	0.01	0.92	
Lateral Lip	**15.4**	**0.0002**	**0.19 (0.04-0.37)**
Lateral Collar	**5.15**	**0.027**	**0.056 (0-0.24)**
Lobes			
Vertical (Alfa)	0.68	0.41	
Medial (Beta)	0.47	0.49	

Table 2.3. F statistic, p-values and effect sizes (partial omega squared ω_p^2) of a general linear model interaction term between type of ant (leaf or trunk) and colony size-related traits (factor 1 of a PCA constructed with these variables: number of spines, percentages of task-specialization and estimated number of outside workers, see Methods). Significant p-values after correction for false discovery rate are shown in bold. Confidence intervals for the estimated effect sizes are shown in parentheses.

Colony code	Spines on host tree	Leaf-marked ants	Trunk-marked ants	Total marked ants
35	5	25	67	92
37	5	44	30	74
31	8	50	45	95
33	8	60	60	120
13	12	51	70	121
38	13	70	70	140
30	15	90	80	170
36	23	60	60	120
42	26	83	70	153
01	27	80	90	170
34	30	161	140	301
45	40	90	72	162
44	41	100	100	200
41	59	135	110	245
40	60	100	100	200
25	61	90	80	170
43	113	75	80	155

Table 2.4. Number of acacia ants (*Pseudomyrmex spinicola*) that were color-marked on either leaves (leaf-marked) or trunk base (trunk-marked) on the acacia host-tree. Because acacia ants obligatorily nests inside hollow spines on the host tree, the total number of spines per host tree is also indicated.

Trait	Loadings Factor 1
Estimated number of outside workers	0.593
Number of spines on the tree	0.577
Defense specialization	0.544
Foraging specialization	0.138

Table 2.5. Loadings of the size-related variables of the acacia ant colonies for the first factor of a principal component analysis, which explained 56% of the variation in the colonies.

	Estimate (log-odds)	SE	Wald	P-value
β_0 Intercept	0.28	2.3	0.014	0.90
β_1 Ant type (reference is trunk-ants)	-1.65	3.3	0.242	0.62
β_2 % Defense specialization	-0.008	0.03	0.085	0.77
β_3 Ant type*defense specialization	0.024	0.04	0.372	0.54

Table 2.6. Estimated parameters of Wald statistic and associated probability (P) for the generalized estimating equation assessing the **effect of defense specialization on the log odds of discarding food** (log odds of discarding food = β_0 (intercept) + β_1 Ant type+ β_2 Defense specialization + β_3 Ant type*Defense specialization). For trunk-ants, the log-odds of discarding changed with task-overlap by β_2, while for leaf-ants they changed by $\beta_2 + \beta_3$.

	Estimate (log-odds)	**SE**	**Wald**	**P-value**
β_0 Intercept	5.60	2.94	3.6	0.056
β_1 Ant type (reference is trunk-ants)	-3.99	1.78	4.9	**0.026**
β_2 % Foraging specialization	-0.040	0.03	1.5	0.217
β_3 Ant type * % Foraging specialization	0.038	0.02	2.5	0.110

Table 2.7. Estimated parameters of Wald statistic and associated probability for the generalized estimating equation assessing the **effect of foraging specialization on the log odds of attacking intruders** (log odds of attacking intruders = β_0 (intercept) + β_1 Ant type+ β_2 Foraging specialization + β_3 Ant type* Foraging specialization).

	Estimate log-odds	**SE**	**Wald**	**P-value**
β_0 Intercept	2.98	3.16	0.89	0.35
β_1 Ant type (reference is trunk-ants)	-4.47	3.16	1.99	0.16
β_2 % Defense specialization	-0.005	0.04	0.02	0.88
β_3 Ant type * % Defense specialization	0.041	0.04	1.24	0.27

Table 2.8. Estimated parameters of Wald statistic and associated probability (P) for the generalized estimating equation assessing the **effect of defense specialization on the log odds of attacking intruders** (log odds of attacking intruders = β_0 (intercept) + β_1 Ant type+ β_2 Defense specialization + β_3 Ant type * Defense specialization).

Chapter 3: Defense of host plant against encroaching vegetation by mutualistic acacia ants

INTRODUCTION

The evolution, stability and maintenance of a mutualism is shaped by benefits that mutualistic partners provide to each other (Bronstein 1998; Sachs et al 2004; Archetti et al 2011). Protective ant-plant mutualisms are among the most widely studied mutualisms, in which plants offer food, shelter or both to the ant colony, and the ants defend the plant against herbivores and encroaching vegetation (Davidson 1993; Heil and McKey 2003). Typically, each plant species associates with more than one ant species, which often vary in the magnitude and efficiency of protection against herbivores. Protection against herbivores is typically used to qualify the benefits provided by different ant species: species that quickly detect and remove herbivores presumably benefit the plant more than species that take longer to identify or and cope with intruders (Rosumek et al 2009; Chamberlain and Holland 2009; Trager et al 2010). However, to understand the evolution and maintenance of protective ant-plant mutualisms, it is necessary to study other ant behaviors that may benefit host plants (e.g., Frederickson 2005). Ants in obligatory ant-plant associations often show an understudied behavior that has consequences for the growth and reproduction of the host tree: reducing host competition by killing encroaching vegetation (Table 3.1).

Neotropical acacia ants (in the genus *Pseudomyrmex*) are well suited for comparative studies of plant killing, because several sympatric, mutualistic and closely-related ant species (*P. spinicola*, *P. flavicornis* and *P. nigrocinctus*) associate with the same plant host-species. All these three ant species are similarly effective in detecting and removing potential herbivores from the host tree (Cronin 1998), but they vary in the area around the host tree where the ants remove competitor plants by pruning (Fig. 3.1 a-

c). *Pseudomyrmex spinicola* ants kill neighboring plants to create vegetation-free areas (hereafter "clearings") with a radius of up to two meters around the host plant (Fig 3.1a), whereas the other two species (*P. nigrocinctus* and *P. flavicornis*) make clearings with a maximum radius of 30 cm (SAV, this study; Fig 3.1b, c).

Acacia ants kill non-host plants by biting plant tissue with their mandibles; the ants either defoliate the plant until it dies or sever the main apical meristem. Interspecific variation in the size of the clearing that ants maintain around the host tree could be an indication of intrinsic differences in the pruning behavior of workers. That is, clearing size could indicate levels of aggression towards plants. For example, workers from species that prune larger clearings may differ in the thickness of the tissue they damage, or in the plant structures they attack, when compared to species that maintain smaller clearings.

Anatomical modifications that yield more force in the mandibles could also explain differences in clearing-area among species, because mandibular force constrains the plant tissue that workers can prune, or the type of plants they can attack and kill. Specifically, head broadness can influence mandible force (Gronenberg et al 1997), which is important for both pruning and defense against intruders. Assuming similar mandible shapes, the movement generated by mandible muscles is related to the angle of attachment inside the head capsule: wider heads allow an attachment angle of the muscle fibers that increases force, whereas elongated heads facilitate attachment angles that increase speed and allow fast mandible strikes (Gronenberg et al 1997; Paul and Gronenberg 1999). Therefore, in ant species that kill vegetation by pruning, wider heads could permit cutting harder tissue, and wider heads therefore could require fewer workers to kill a plant. These considerations predict that wider heads (maximizing mandibular

force) for acacia ants species that make the largest clearings or that prune the hardest plant tissues.

Here, I assessed whether large-scale interspecific clearing patterns correlated with fine scale behavioral choices and head broadness. I evaluated whether workers from three acacia ant species (*P. spinicola*, *P. flavicornis* and *P. nigrocinctus*) that make clearings of different size, also vary in the plant structures they prune, specifically testing the prediction that the ant species that prunes the most (*P. spinicola*) should have wider heads than the two ant species with smaller clearings. The anatomical comparison also included three non-pruning species: *P. viduus*, which lives in mutualism with *Triplaris melaenodendron* trees; *P. nigropilosus* and *P. gracilis,* two species that parasitize the acacia tree by nesting and feeding on it but do not protect it against herbivores or vegetation. I expected all non-pruning species except *P. gracilis* to have elongated heads compared to pruning mutualistic species. *P. gracilis* is a predator that relies on mandible force to hold the prey and sting it, so I expected this species to have heads as wide as species pruning hard plant tissue.

MATERIALS AND METHODS

Study site and species

The study was carried out at Palo Verde National Park (10° 21' N, 85° 21' W) in Guanacaste, Costa Rica, near the facilities of the Organization for Tropical Studies, a dry forest with secondary growth. *Vachellia collinsii* (formerly *Acacia collinsii*) is the most common acacia tree, which can be inhabited by a single colony of mutualistic *Pseudomyrmex* ants (*P. spinicola*, *P. nigrocinctus* or *P. flavicornis*) that prune and kill neighboring vegetation with different intensities (Fig. 3.1a-c).

Clearing size

I sampled the first 10 observed acacia trees in association with one of the three mutualistic species of ants, until I had 30 trees in total (*P. flavicornis*, *P. nigrocinctus* or *P. spinicola,* n = 30). For a tree to be sampled it had to be at least 4 m away from similar-sized acacia trees, to ensure that the measured clearing had been created by the workers of only one colony. I measured the host tree diameter at the base of the trunk and the radius of the clearing (i.e., vegetation-free area) at the four cardinal points. A general linear model was fitted to the data to analyze the effect of ant species (fixed factor) on the mean clearing radius (response variable, square-root transformed to meet assumptions of the analysis), controlling for tree diameter (continuous covariate) which is also related to colony size.

Individual pruning behavior by ant workers

For species comparisons, I quantified the pruning behavior of mutualistic acacia ants on an herbaceous plant (the grass *Oplismenus sp.*) and on saplings of a woody species (*Lysiloma divaricatum*). Grass bunches (with stalks of 7-8 fully-expanded leaves) and tree saplings (approx. 15 cm height) were planted in plastic bags (henceforth, experimental plants). I observed experimental grasses placed next to 48 acacia trees inhabited by *P. spinicola*, 22 trees inhabited by *P. flavicornis*, and ten trees associated with *P. nigrocinctus*. Pruning observations on *L. divaricatum* saplings occurred in 12 colonies of *P. spinicola*, 12 colonies of *P. flavicornis* and eight colonies of *P. nigrocinctus* (one ant colony = one acacia tree). All colonies were sampled once for the grass or woody sapling, and each experimental plant was used only once.

Experimental plants were placed on the ground touching the acacia tree by the second leaf and not by the youngest and softest first leaf. The position where ants pruned the grass was recorded by node, which were numbered in ascending order from top to

base of the grass (e.g. seventh node is older than fifth node; Fig. 3.4). To simplify the nomenclature, I have called "nodes" the points on the stem at the level of the leaf laminas or ligules, although technically nodes on grasses are covered by the sheathing petiole and located lower than the ligule. To record pruning on tree saplings, which had compound leaves, I noted whether ants were biting into (1) leaflets, (2) apical meristem, (3) leaf mid-vein or (4) the main stalk and branches. After the experimental plants were placed next to the acacia tree, I observed them for 30 minutes and recorded the places chewed by the ants. The reported pruned nodes correspond to nodes where we saw workers biting even if they were unsuccessful in severing the plant by that part.

To determine the degree of variation in the stem diameter presented to the ants, experimental plants were measured with a caliper (accuracy of $\pm$ 0.01 mm). For the grasses, I measured the diameter of the stem at each node. Because ants usually cut grasses while supporting themselves on the grass stalk, I also measured the distance between nodes (Fig. 3.4). Mean $\pm$ SD values are presented. I used a one-way ANOVA to analyze the effect of the grass node position (fixed factor) on the diameter of the stem or the distance between nodes, and each plant was included in the model as a block (random factor). Fisher's LSD post-hoc comparisons were performed. In the shrub saplings, I measured the diameter of the principal stem at each node, at the base of the branches, and at the petiole of each leaf. To test the effect of node position (fixed factor) and the effect of the plant part (petioles and main stem, fixed factor) on the diameter of the tissue I run separate one-way ANOVA analyses. For both analyses I used the individual plant as a random factor (block). Fisher's LSD post-hoc comparisons were made between nodes on the stem or among leaf positions.

Head morphology

To assess whether higher pruning intensity was associated with wider heads, I studied the head morphology of seven acacia ant species. For the species that prune vegetation I studied the three acacia mutualists mentioned above (48 *P. spinicola* ants, 23 *P. nigrocinctus* and 20 *P. flavicornis*); and *P. satanicus* (90 ants) inhabiting *Vachelia melanoceras* from Parque Nacional San Lorenzo, Panamá (9°19' N, 80°0' W). *Pseudomyrmex satanicus* is the sister species of *P. spinicola* and also kills vegetation near the host tree (Ward 1990; Ward and Downie 2005b). For the non-pruning species, I studied *P. nigropilosus* (25 ants), a species that obligatorily nests in acacia trees but does not protect it against herbivores or prunes vegetation; its sister species (*P. gracilis,* 21 ants), which is a predator that facultatively can nest on acacia trees; and *P. viduus* (15 ants), which lives in association with *Triplaris melaenodendron* trees but are not known to prune or kill vegetation.

I took dorsal pictures of the heads with a Leica camera attached to a dissecting scope, and measured head length and width using ImageJ (Fig. 3.5; Schneider et al 2012). Specimens of *P. satanicus* were directly measured under the stereomicroscope with a micrometer at the facilities of Smithsonian Tropical Research Institute. I then calculated the ratio of head length to head width (hereafter, "head broadness"). For this measure, a value of one means that the head is rounded; values larger than one correspond to elongated heads, and values of less than one correspond to widened heads.

I tested head broadness for phylogenetic signal with the 'phylosig' function of the 'phytools' R package, using the Blomberg K and 10000 repetitions. I reduced the phylogenetic tree of Gómez-Acevedo et al (2010b) to include only the species used in this study. Head broadness was independent of phylogenetic position ($K = 0.339$, $p = 0.45$), so I performed all statistical analysis without phylogenetic correction. The

statistical models included two independent factors: reaction towards vegetation (pruning vs. non-pruning, fixed effect) and the type of association with the plant (tree mutualists vs. non-mutualists); and the response variable of head broadness (log transformed to meet normality assumptions).

RESULTS

Clearing size

The size of the clearing depends on the species of ant. Clearings of *Pseudomyrmex flavicornis* and *P. nigrocinctus* were similar in size (23.62 ± 13.2 and 19.8 ± 3.11 cm, respectively; T = 0.65, p = 0.52), but *P. spinicola* workers produce clearings about five times larger than clearings of *P. flavicornis* or *P. nigrocinctus* (mean ± SE: 108.8 ± 10.3 cm; Fig. 3.1; T = 6.12, p < 0.0001).

Individual pruning behavior by ant workers

All three acacia mutualist ants bit on one particular node on the grass more often than other nodes: *P. spinicola* cut most *Oplismenus* grasses on the third leaf blade (Fig. 3.2; G test, $X^2 = 12.9$, d.f. = 6, p = 0.04), whereas *P. flavicornis* preferentially pruned the youngest nodes (G test, $X^2 = 14.2$, d.f. = 6, p = 0.03) and so did *P. nigrocinctus* (G test, $X^2 = 26$, d.f. = 6, p < 0.0001). Two colonies of *P. nigrocinctus* (out of 10 colonies) and two of *P. flavicornis* (out of 22) did not prune the experimental grass during the observation time. In *P. nigrocinctus*, only one colony pruned on more than one node; the rest of the colonies left the plant and returned to the acacia tree after the first leaf blade of the grass collapsed. The nodes on the *Oplismenus* grass all had different diameters ($F_{6, 138} = 43.4$, p < 0.0001): oldest (seventh) and intermediate nodes (fourth and fifth) had the greatest stem diameters, while the first node was the thinnest (Fig. 3.4a). The greatest change in average diameter was from the first to the second node (an increase of 0.4

mm). Distance between nodes varied between 2-25 mm, and increased from the top to the base of the grass (Fig. 3.4b; $F_{4, 92} = 119.02$, $p < 0.0001$). All distances between nodes were different from each other (Fisher LSD post hoc test, all $0.05 < p < 0.0001$), and increased toward the base of the grass. However, the distance between nodes was not relevant in pruning decisions, because all species pruned on the second node, which had the shortest distance to the following node.

When woody saplings were used as the experimental plants, all three species of ants mostly pruned leaflets by biting the petiolules, which were 0.01 mm in diameter. In *P. spinicola* pruning of mid-veins was more common than in *P. flavicornis*, whereas *P. nigrocinctus* ants never attempted to cut leaf mid veins. *P. spinicola* was the only species that cut branches or the main stem (Fig. 3.3). Diameter of the leaf mid-veins (0.35 ± 0.16 mm) was similar between leaves on different nodes ($F_{4, 17} = 2.12$, $p = 0.12$), and smaller than the average diameter of the principal stem (0.80 ± 0.42 mm, $F_{1, 50} = 39.8$, $p < 0.001$).

Head morphology

The variation in head broadness of the ants was affected by type of association with the plant (mutualist vs. non-mutualists, $F_{1, 229} = 13.76$, $p < 0.0001$), as well as by the pruning behavior (pruning vs. non-pruning, $F_{1, 229} = 80.42$, $p < 0.0001$). As expected, non-mutualistic ants had more rounded heads than mutualistic ants, and pruning ants had more rounded heads than non-pruning ants, when controlling for type of association (Fig. 3.5). Among the species that prune, *P. spinicola* has the broadest heads, and their heads are almost as broad as those of *P. gracilis*, the predator species. The heads of *P. satanicus* were more elongated than those of the sister species *P. spinicola*, although they both kill nearby vegetation in large areas around the host tree. The two other species that prune

and make smaller clearings (*P. nigrocinctus* and *P. flavicornis*) had even more elongated heads, and finally, *P. viduus* was the species with the most elongated head (Fig. 3.5).

DISCUSSION

In several protective ant-plant mutualisms, workers are aggressive against neighboring vegetation (Table 3.1) to reduce competition with the host plant. Here, I showed that variation among species in the area around the host plant cleared from vegetation, was related to workers making different decisions on the thickness of the tissue they prune. Specifically, the ant species that maintained the largest vegetation-free areas (*P. spinicola*) tended to cut the thickest tissue. Correspondingly, the other species cleared less area of vegetation, and pruned the thinner parts of the plants. Larger clearing size and thickness of pruned tissue correlated with workers having broader heads, which is related to mandible force. Head broadness may facilitate pruning in acacia mutualists, and our findings indicate that selection may act to favor broader heads that allow the generation of greater mandible force.

Ecologically, explanations for the interspecific variation in pruning intensity (cleared area, thickness of plant tissue) can be generated from the plant perspective or from the ant-colony perspective. For instance, interspecific pruning variation among *Crematogaster* (*Decacrema*) species inhabiting different species of *Macaranga* trees was partially explained by the risk of invasion to the resident colony from other ants: *Crematogaster* species that prune the most, inhabit *Macaranga* species that lack a waxy stem, which prevent intruder ants from walking onto the slippery host tree (Federle et al 2002). However, unlike *Crematogaster* in *Macaranga*, pruning variation among closely related species of acacia ants is less likely caused by variation vulnerability to invasions (i.e., variation in access to the host tree), because acacia trees do not have a barrier such

as waxy surfaces towards intruders. In acacia ants, tree traits related to growth and vegetative reproduction are more likely to explain pruning variation. Although all species studied here can establish colonies on *V. collinsii* acacia trees, most of these trees are inhabited by the ant species that prunes the most. A second sympatric species of acacia (*V. cornigera*) is usually inhabited by *P. flavicornis* and *P. nigrocinctus*. In *V. collinsii* trees the vegetation-free area created by the ants enhances the establishment of acacia sprouts which are colonized by the ant colony that created the clearing (Amador-Vargas 2012a). If the two tree species vary in the benefits they obtain from pruning, e.g. because they have different shade-tolerances or reproduction by rhizomes, the co-occurrence of the tree species could be maintaining low-pruning and high-pruning ant species in the same area. To assess this hypothesis, variation in shade-tolerance or asexual reproduction between the two acacia species will need to be documented.

The area clear from vegetation around the host tree was associated with individual decisions about pruning sites, which I argued could be associated with head shape and mandible force. Broader heads allow for a greater attachment angle of the mandible muscles which generates greater force (Paul and Gronenberg 1999). In my study, head shape varied as predicted from observed pruning intensity: *P. spinicola*, the species that prunes the most (largest clearing, thickest tissue attached; Fig. 3.5) had also the broadest head among acacia ant mutualists. The sister species, *P. satanicus*, had the second broadest head and although I lack behavioral observations of this species, workers prune and maintain clearings that are a similar size to those of *P. spinicola* (SAV pers. obs.).The head broadness of mutualistic acacia ants has diverged since they shared an ancestor with *P. viduus*. *Pseudomyrmex viduus* has a protective ant-plant mutualism with *Triplaris* trees but does not prune or clips Beltian bodies from the tree as acacia ants do. On the other hand, *P. gracilis*, the acacia ant species with the widest heads, are strong

predators that kill by biting and stinging, whereas the head of its sister species *P. nigropilosus* is more elongated, even more elongated than that of *P. spinicola*, the mutualistic species that prunes the most (Fig. 3.5). In addition, *P. nigropilosus* ants have a similar nesting- and foraging-habit to mutualistic acacia ants, but they differ in their aggressiveness against vegetation and also in head broadness (Janzen 1975; Amador-Vargas 2012b). In sum, head broadness seems to be a plastic trait that presents a significant amount of variation among closely related acacia ant species. Further studies on the behavior and head shape of other pruning ant-species and non-pruning sister taxa (examples in Table 3.1) would provide more evidence to test the hypothesis that selection for pruning behavior selects also for broader heads and greater mandibular force. A further prediction of this hypothesis is that broader heads would not be expected in ants that kill neighboring vegetation by injecting poison, such as *Myrmelachista* ants (Frederickson et al 2005). Studies on the internal anatomy of the heads of these ants could strengthen our conclusions and would also allow evaluation of selective pressures on other structures inside the head capsule (e.g. brain and glands).

In protective ant-plant mutualisms, studies often focus on how potential partners for the plants vary in the defense against herbivores, while the protection against encroaching vegetation is often understudied. Even in acacia ants, one of the most studied associations, we know very little about pruning behavior and the potential causes of behavioral variation among species. This study links variation in area cleared of vegetation among species with differences in decision-making about pruning sites, which could be presumably explained by mandible force.

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TABLES

Ant partner	Host plant	Pruned plants: Vines	Pruned plants: Nearby vegetation	References
Neotropics				
Azteca	*Cecropia*	yes	no	(Janzen 1969; Schupp 1986; Davidson, Longino, and Snelling 1988)
Allomerus demararae	*Cordia nodosa*	yes	yes	(Davidson, Longino, and Snelling 1988)
Myrmelachista	*Duroia/ Tococa/ Clidemia*	?	yes	(Morawetz, Henzl, and Wallnofer 1992; Renner and Ricklefs 1998; Frederickson, Greene, and Gordon 2005)
Pseudomyrmex dendroicus	*Triplaris americana*	yes	yes	(Davidson, Longino, and Snelling 1988)
Pseudomyrmex triplarinus	*Triplaris americana*	?	yes	(Larrea-Alcázar and Simonetti 2007)
Pseudomyrmex ferrugineus group (10 spp)	*Acacia*	yes	yes	(Janzen 1966; Janzen 1974; Janzen 1991; Amador-Vargas 2012a)
Pheidole bicornis	*Piper*	yes	?	(Risch et al. 1977)
Paleotropics				
Crematogaster	*Macaranga*	yes	yes	(Fiala et al. 1989; Federle, Maschwitz, and Holldöbler 2002)
Tetraponera aethiops	*Barteria fistulosa*	?	yes	(Yumoto and Maruhashi 1999)

Table 3. 1. **Obligate plant-mutualistic ant species that prune encroaching vines or vegetation growing on the vicinity of their host tree**. Species reported to prune are listed as "yes", when not pruning as "no"; a question mark indicates that no explicit record of pruning behavior was found in the literature.

FIGURES

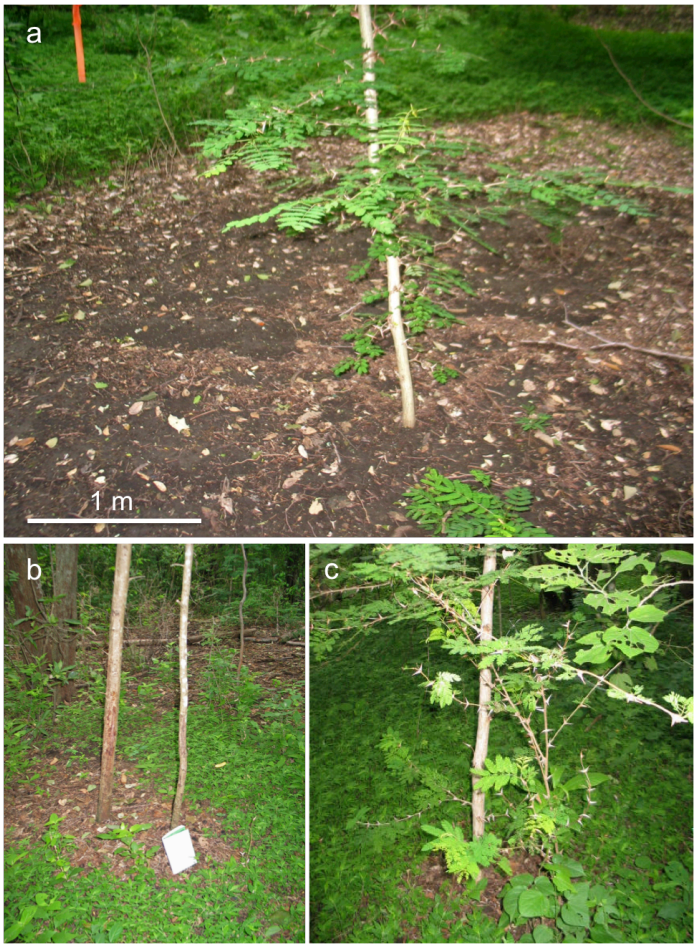

Figure 3.1. Ants associated with acacia trees prune vegetation in the vicinities of their host plants that create clearings on the ground. The size of the clearing varies among species: (a) *Pseudomyrmex spinicola* colonies make clearings about five times larger than those of (b) *P. flavicornis* or (c) *P. nigrocinctus*. Pictures show the main trunk of an acacia tree, surrounded mostly by the grass *Oplismenus* sp.

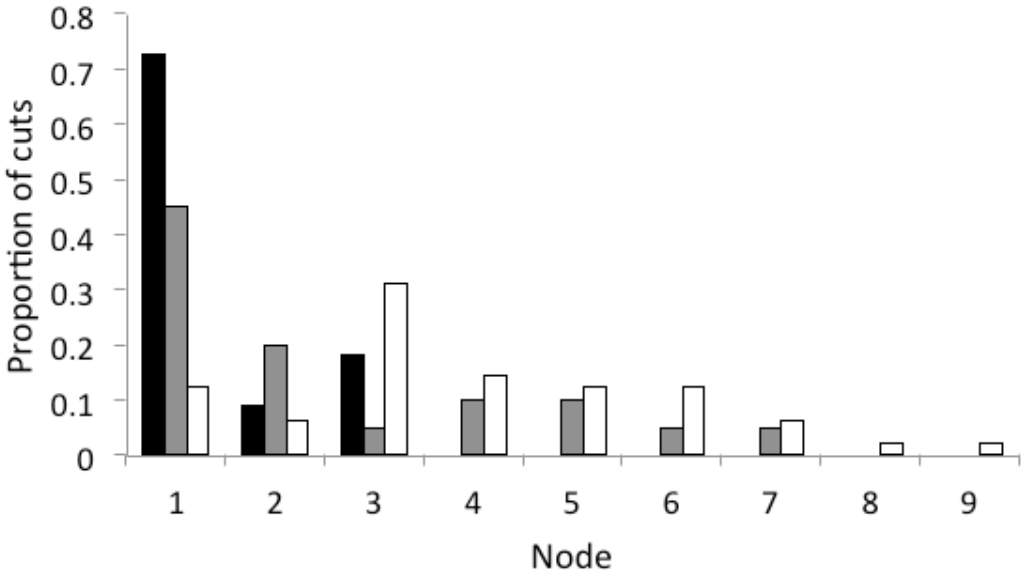

Figure 3. 2. Proportion of nodes that workers pruned on grass by the ants *Pseudomyrmex nigrocinctus* (black bars, n=11 workers observed), *P. flavicornis* (gray bars, n= 20) or *P. spinicola* (white bars, n= 46). Nodes of a grass stalk are numbered increasingly from the apical meristem to the base of the grass (e.g., node 1 was the node supporting the youngest leaf of the grass).

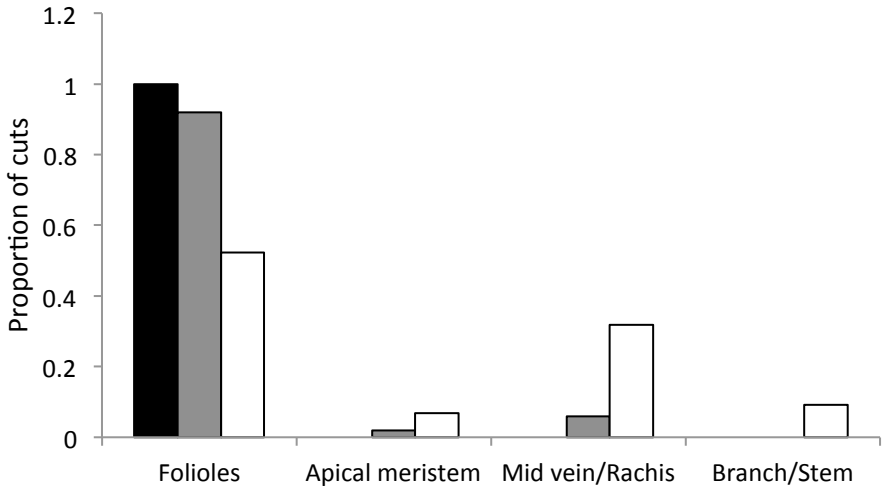

Figure 3. 3. Proportion of bites by acacia ants on various plant parts of *Lysiloma divaricatum* saplings placed next to trees inhabited by *Pseudomyrmex nigrocinctus* (n = 28, black bars), *P. flavicornis* (n = 53, gray bars), or *P. spinicola* (n = 56, white bars).

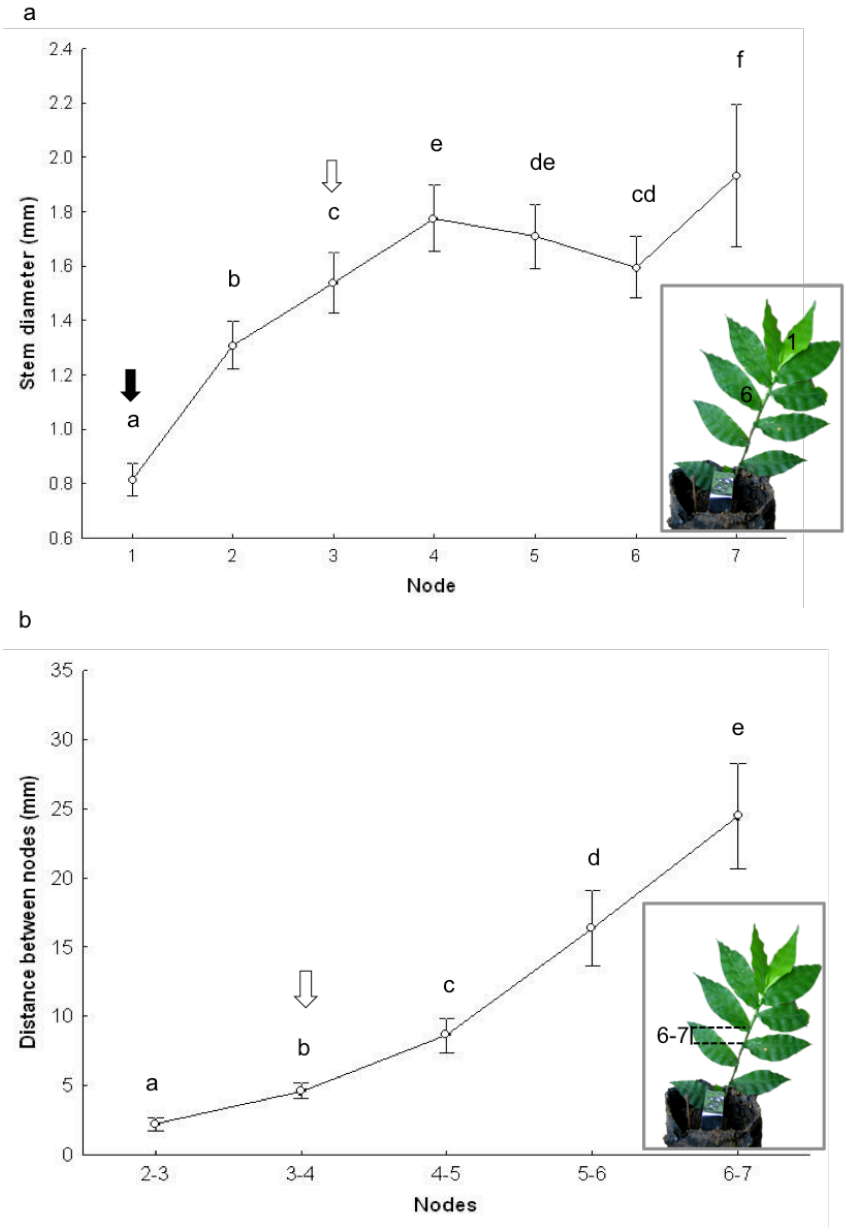

Figure 3. 4. (a) Stalk diameter of the *Oplismenus* grass as a function of leaf age, ranging from the partially unfolded youngest leave (node 1) and at subtending older leaves (node numeration shown in inset). **(b)** Distance to node of next-older leaf (the inset shows the inter-node distance between leaves 6 and 7)). Circles represent the averages; error bars denote 0.95 confidence intervals. Letters denote statistically different groups ($p < 0.05$, ANOVA test, Fisher's LSD comparisons). Arrows represent the most pruned point by *P. spinicola* (white) or by *P. flavicornis* and *P. nigrocinctus* (dark).

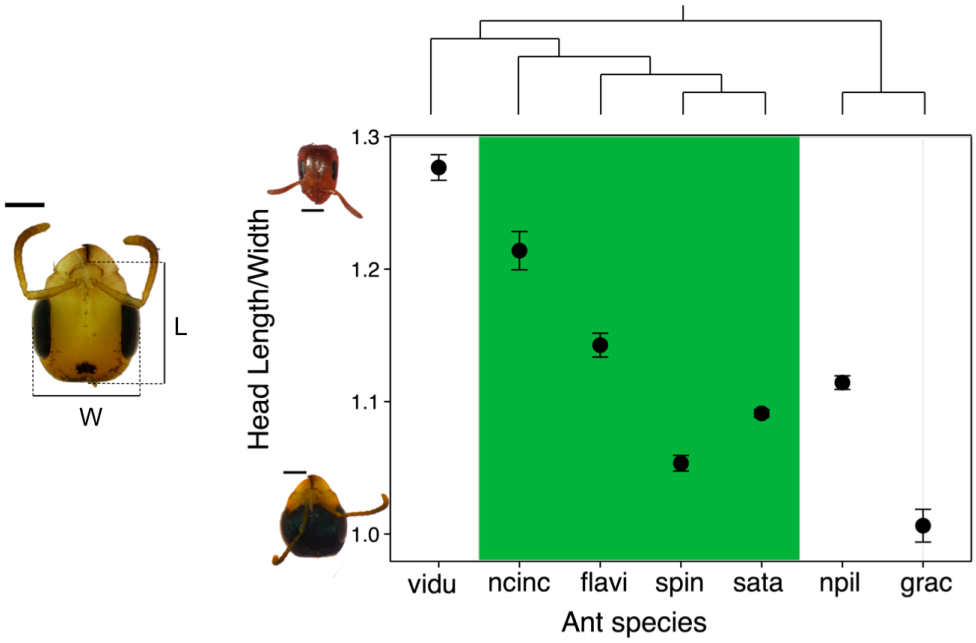

Figure 3. 5. **Head broadness measured as the ration of head length (L) over head width (W) in four obligatory acacia ants that prune vegetation** (shaded area): *P. nigrocinctus* (ncinc), *P. flavicornis* (flavi), *P. spinicola* (spin) and *P. satanicus* (sata); **and three non-pruning species**: *P. nigropilosus* (npil, head indicating measurements is shown in the picture), *P. gracilis* (grac), and *P. viduus* (vidu), a species associated to *Triplaris* trees. Circle represents the mean and whiskers the standard error. Heads with small ratios are broad (such as the head of *P. gracilis* shown near 1.0 in the y-axis), whereas higher values indicated elongated heads (such as the head of *P. viduus* shown near 1.3 in the y-axis). The phylogenetic relationships between the ant species are depicted on top with illustrative purposes (i.e. length of branches does not represent phylogenetic distance). Scale bars for all images is 0.5 mm

Chapter 4: Orientation in ants lacking central-place foraging

INTRODUCTION

Returning to a particular place to provision offspring –called central-place foraging– relies on spatial orientation abilities, which are key in the survival and reproduction of animals (Collett et al 2013). Social insects in the order Hymenoptera (i.e. wasps, bees and ants) are typically central- place foragers; some species show striking orientation abilities and are able to exploit ephemeral resources located hundreds of meters away from their nest, which is often inconspicuous (Beekman and Ratnieks 2000; Steck et al 2009; Zurbuchen et al 2010). Hence, life history and ecological demands directly influence cognitive abilities of animals engaged in solving problems of central-place foraging central-place foraging.

Orientation studies have traditionally focused on questions how ecological demands cause animals to excel at highly demanding navigational tasks. For instance, bird species that store food have been tested for spatial memory to compare them with closely-related non-storing species (Balda and Kamil 1989; Bednekoff et al 1997). However, very few studies have assessed how the orientation abilities of animals change with decreased navigational demands. Ants in obligate and permanent associations with plants are well suited for elucidating navigation in species that have in-nest foraging instead of central-place foraging, and therefore decreased navigational demands.

All ants evolved from central-place foragers (Collett et al 2013), but mutualistic, obligate associations with plants can change the foraging habits of an ant species. In ant species nesting exclusively on specific plants, foraging dynamics change from central-place to "in-nest" foraging. Plants in obligate mutualisms with ants usually provide spaces where the ants nests and all the food that the colony requires, and workers therefore do not need to leave the plant to forage (Hanson and Longino 2006; Rico-Gray

and Oliveira 2007). When foraging ecology changes from central-place foraging to in-nest foraging, we may expect evolutionary change in cognitive abilities because in-nest foraging does not require the costly investment in expensive neurological tissue needed for spatial orientation of a typical ant (Laughlin et al 1998; Niven and Laughlin 2008).

Although workers in obligate plant associations do not forage outside the nest, other behaviors can also have high orientation demands. In obligate ant-plant mutualisms, workers still need to orient back to the tree when they leave to defend it against intruders or to kill encroaching vegetation by pruning (Morawetz et al 1992; Renner and Ricklefs 1998; Frederickson et al 2005; Amador-Vargas 2012a). In plant-associated ants, the behavior of pruning nearby vegetation, and the distance away from the plant that workers travel to do so, may enhance their orientation abilities, counterbalancing the lower navigational demands of in-nest foraging.

Acacia ants (*Pseudomyrmex sp*.) are a classical example of coevolution and obligatory mutualism with acacia trees (Janzen 1966; Janzen 1974). Acacia ant workers exclusively feed on the nectar produced on nectaries of all leaves, and feed the larvae with protein-rich food bodies (so-called Beltian bodies) that the host-tree grows on the tips of young leaves for the sole purpose of feeding the ant brood. Ants nest inside the tree's swollen and hollow spines, where workers attend the queen and brood, and store Beltian bodies. Workers only leave the acacia tree (i.e., the nest) to kill neighboring vegetation by pruning, and to patrol neighboring acacia seedlings for possible colonization (Janzen 1966). Species of acacia ants vary in the area they keep clear from non-acacia vegetation, that is, in how far workers travel away from the host tree (Fig. 4.1; Amador-Vargas *in. prep*.; Janzen 1966).

Comparative studies of orientation in animals are difficult because species differ not only in foraging behaviors but also in many other aspects of their life history; some

species can have unique behaviors with no equivalent in the other species, or they can live in very different environments. Acacia ants have closely-related species that are typical central-place foragers, which also nest on acacia trees and occur at the same sites (*P. nigropilosus* and *P. gracilis*; Ward 1993), although they have a parasitic association with the tree. *Pseudomyrmex nigropilosus* ants obligatorily nest on acacia trees and exploit the plant rewards without providing protection against herbivores (Janzen 1975); workers also forage off the host plant and extract food rewards from neighboring acacia trees that are inhabited and protected by mutualistic species (Amador-Vargas 2012b). *Pseudomyrmex nigropilosus* workers, therefore, must travel back to the nesting tree to store the stolen food. The sister species of *P. nigropilosus* is *P. gracilis* (Ward and Downie 2005), a species that facultatively nests on the swollen spines of acacia trees, where it uses the nectar rewards; workers also forage off the host tree to prey on small arthropods to feed the larvae, relying less on the acacia Beltian bodies as protein source (pers. obs.; Clement et al 2008). These two central-place forager species allow for a comparison between central-place foragers and in-nest foragers that nests on similar trees.

In this study, we compared the orientation of three mutualistic acacia ant species (*P. nigrocinctus*, *P. flavicornis* and *P. spinicola*), and of two other species that also nest on acacia but that are typical central-place foragers like most ants (*P. nigropilosus* and *P. gracilis*). In the field, we experimentally disoriented workers and measured several behaviors when workers were trying to return to their host tree. If the mutualism with the plant changed the evolution of orientation abilities of workers, then, we expected workers from the in-foraging species to be more disoriented than control workers by our experimental manipulations. In contrast, workers of the central place foraging species should be less (or not) disoriented compared to control workers. We also tested for an

effect of the distance that workers travel away from the host tree on the disorientation test; we expected workers to differ between control and disorientation trials in the mutualistic acacia ant species that travel the shortest distance away from their host tree *P. nigrocinctus* and *P. flavicornis*, compared to workers of *P. spinicola* that travel longer distances away from the host tree to prune neighboring vegetation (Fig. 4.1 a-c).

MATERIALS AND METHODS

Study site and species

The study was carried out in 2012 and 2013 at Palo Verde National Park (10° 21' N, 85° 21' W) in Guanacaste, Costa Rica, near the facilities of the Organization for Tropical Studies. Palo Verde National Park protects a dry forest with secondary growth (1500 mm mean annual rainfall) where acacia trees *Vachellia collinsii* (formerly, *Acacia collinsii*) are abundant. An acacia tree is usually inhabited by a single colony of ants with one queen.

Several ant species in the genus *Pseudomyrmex* are inhabit acacia plants and occur in Palo Verde: (a) three species of mutualistic ants, *P. spinicola*, *P. nigrocinctus* and *P. flavicornis*, all three of which defend the tree against potential herbivores and kill vegetation growing around the host tree by pruning, producing a circular clearing (Fig. 4.1 a-c); and (b) two species of *Pseudomyrmex* ants that are parasites of acacia trees, that is, colonies of these species nest on the tree but do not defend it. The two parasitic species are: (1) *P. nigropilosus* which only nest on acacia trees and regularly forages off the host tree to obtain food from neighboring ant-defended acacia trees (Amador-Vargas 2012b); and (2) *P. gracilis* which facultatively nests on acacia trees (i.e., colonies of this species can be found nesting in hollow twigs of other tree species) and which

complements it diet by preying on small arthropods that captures on the tree. Based on this information, we categorized the mutualistic species as within-foraging species and the two parasitic species as central-place foragers (Table 4.1).

Orientation assays

To compare the orientation abilities of the five species of acacia ants, we conducted orientation assays in the field. We placed food on the center of a disc on the ground, 20 cm away from the host tree (Fig. 4.2). The disc had two strings attached to the ground-facing side, which could be pulled to rotate the disc either clockwise or counterclockwise. A white stripe marked on top of the disk was initially aligned with the point where ants, coming from the trunk of their host tree, walked onto the disc at the beginning of the experiment (Fig. 4.2). A Beltian body was placed into the center of the disc to motivate a worker finding this food item to return to the host tree and begin the return trip by orienting itself. When an ant worker picked up the food item with its mandibles, we rotated the disc 45° clockwise or counterclockwise. Because we were interested in disorienting the workers, the discs had a line painted across (Fig. 4.2). This line was a conspicuous visual cue that if workers on rotated treatments followed on their way back to the acacia they would be disoriented; that is, following the line would have led them to a point other than the host tree. As control trial, we rotated the disc first about 20°, then back to the original position (white stripe aligned with the direction of the host tree). This control manipulation controlled for the motion of the disc moving without changing the visual cues at the end of the back-and-forth motion from those at the beginning (i.e., if workers used visual cues to follow the white line on the disc, the line would lead the worker to the point on which they entered the disc).

To motivate a return trip in all species except *P. gracilis*, we baited the ants with Beltian bodies freshly cut from the host tree as food; we used dead mosquitoes as bait for the predatory *P. gracilis* workers. Offering different types of food to different ant species is unlikely to affect our results because our analysis only compares control and rotated ants of the same species. We sampled one to three ants per host-tree, making sure they were different workers; i.e., a new worker entered the disc while we could still see the earlier workers walking with the food item on the tree (*P. nigrocinctus* N = 39 workers on xx trees; *P. flavicornis* N = 41 on xx trees; *P. spinicola* N = 35 on xx trees; *P. nigropilosus* N = 28 on xx trees; *P. gracilis* = 28 on xx trees). We consider observations of workers from the same colony to be independent, because foraging in *Pseudomyrmex* is solitary and the behavior of each worker on the experimental disc is independent from the workers in the rest of the colony. *Pseudomyrmex* ants rely heavily on vision for foraging; although, we wiped the disc after each trial with water and tissue; we also rotated several discs to do the experiments. In treatment and control assays, we video recorded the worker's path back to the acacia tree using a Nikon J1 camera capturing videos at 30 frames per second.

We analyzed the videos frame by frame to obtain the response variables. To asses whether workers were leaving the experimental disc in a direction different than towards the host-tree, we quantified the mean angle at which workers where walking with respect to the tree using the software Tracker (Brown 2014). For each worker, we marked the ant position every 10 video-frames (every 0.33 s) according to a coordinate system that positioned the host-tree at 0° with respect to entrance point on the disc (Fig. 4.2), and then we calculated the circular mean of those angles deviating from the direction of the original entry point (i.e., host-tree). To assess whether workers started and ended up with a deviation from a straight line towards the acacia tree (0°), we compared the mean angle

of the worker's trajectory with respect to the acacia tree between control and treatment ants at the beginning of the path (during the first 3.3 seconds after they picked up the food item) and during the entire path back to the tree. Because some discs were rotated clockwise and others counterclockwise, we standardized the coordinate system to have the 0° pointing toward the acacia tree, the positive values up to 180° in the direction of the disc rotation, and the negative values in the direction contrary to the disc rotation (Fig. 4.2). We also calculated the walking speed of workers using the automated function of Tracker software, to assess whether ants would walk more slowly when rotated 45° in one direction versus 20°-and -20° back-and-forth in the control treatment.

We used JWatcher software (Blumstein et al 2006) to count the number of times that ants performed the following behaviors: (1) turning away from the host-tree after reaching the disc edge (Fig. 4.4g); (2) walking off the disc at a point other than the initial entrance point (Fig. 4.5g); (3) time returning to the host-tree; and (4) returning to the disc center, where they first picked up the food reward, after reaching the disc edge (Fig. 4. 8g); (5) number of spins, defined as complete 360° rotation with no displacement (Fig. 4.9g); and (6) number of stops (i.e., temporary absence of movement lasting at least X sec). We could not record behavioral observations blindly because the species of ant and the treatment (disc rotation) were evident in the video-recordings.

Phylogenetic Corrections and Statistical Analyses

We first tested behavioral variables for phylogenetic signal to determine whether our analysis required a correction for phylogeny. We calculated the effect size (Cohen's D) for all variables except for the behaviors of stopping, spinning or walking off the experimental disc at a point different than the entry point, because each test species rarely showed these behaviors, or because there were no differences between control and

treatment condition for any species. Second, we used a phylogenetic tree with only the species included in the study based on Gómez-Acevedo et al (2010). We then tested whether the effect sizes for each behavioral variable had phylogenetic signal by calculating Blomberg's K (Blomberg et al 2003). We also ran a randomization test (10 000 permutations) to assess whether the calculated variance for the independent contrasts was significantly different than expected under random trait variation, in the function "phylosig" on the phytools (Revell 2012) package in R. The values of the walking angle during the entire route cannot be treated as regular continuous variables due to their circular nature. Instead of calculating a numerical effect size, we coded the effect as a binary variable: "1" if the mean angle of control and rotated treatments was different, and "0" if they were not different. For this binary data we calculated the D-value or phylogenetic signal (Fritz and Purvis 2010) using the "phylo.d" function of the caper R package (Orme 2013). Because none of the analyzed traits showed phylogenetic signal (Table 4.2), we therefore treated species as independent in all analysis.

We used a generalized linear model with Poisson distribution for the count data (counts of walking towards the acacia tree on the edge of the disc, of walking towards the center of the disc, and number of stops and spins), or a linear model for our continuous variables (walking speed and time returning to the tree, both log transformed) to test for an interaction between type of foraging (central-place vs. in-nest) and treatment (control vs. rotated). We expected the effect of rotating the disc to depend on whether workers were central-place foragers, which would result in a significant interaction term between type of foraging and treatment. We also tested whether the effect of the treatment was dependent on the distance workers typically travel away from the tree (long- vs. short-distance as assigned in Table 4.1), that is, we tested for the statistical interaction of treatment and traveling distance. Additionally, we analyzed each species separately

assessing the effect of the treatment on the response variable. For circular data (the walking angle during the first 3 seconds, and the walking angle of the entire route) we compared angles of ants on control and rotated treatments with a Watson U^2 test using the function "watson.two.test" from the circular package in R (Agostinelli and Lund 2013). We also tested whether those angles were different from zero (i.e. from pointing towards the acacia host-tree) with a Rayleigh test (Zar 2010). We used chi-squared tests for counts (e.g. number of times returning to the disc center) and Mann-Whitney U tests for continuous data (e.g. speed, time to return to the tree).

RESULTS

Walking angle

During the first 3.3 seconds of walking after the worker picked up the food, mean walking angle of workers was affected in one of the in-nest forager species (*P. nigrocinctus*) and in both central-place foragers. Workers on rotated treatment walked at an angle about 20° larger than control ants in *P. nigrocinctus* (Figs. 4.3a, b; $F_{1, 37} = 3.88$, $p = 0.05$) and central-place foragers of *P. gracilis* (Figs. 4.3i, j; Watson $U^2_{27} = 0.18$, $0.01 < p < 0.05$). Also, the other central-place forager, *P. nigropilosus*, showed a deviation of about 8° with respect to the control (Figs. 4.3g, h; Watson $U^2_{28} = 0.25$, $0.01 < p < 0.05$). Workers on rotated treatment showed no difference to control workers in the other two in-nest forager species: *P. flavicornis* (Figs. 4.3 c, d; $F_{1, 39} = 0.09$, $p = 0.10$) and *P. spinicola* (Figs. 4.3 e, f; $F_{1, 33} = 1.18$, $p = 0.28$).

When considering the entire route, workers on rotated treatment showed deviations from control workers in one of the in-nest foragers expected to get lost easily, *P. nigrocinctus* (Figs. 4.3k, l; Watson $U^2 = 0.23$, $p < 0.05$), but not in the other species (*P. flavicornis*; Figs. 4.3m, n; Watson $U^2 = 0.09$, $p > 0.05$). As expected for central-place

foragers, the initial difference between treatment and control in *P. gracilis* workers was no longer found when considering the entire trajectory (Figs. 4.3s, t; $F_{1,25} = 0.07$, $p = 0.79$). Surprisingly, rotated workers of the other central-place forager *P. nigropilosus*, still differed from control workers in the walking angle when considering the entire route (Figs. 4.3q, r; Watson $U^2_{28} = 0.32$, $0.001 < p < 0.01$). We also found that *P. spinicola* workers, who naturally travel the furthest away from the host tree, performed similarly on rotated and control discs (Figs. 4.3o, p; $F_{1, 33} = 1.78$, $p = 0.19$).

Were workers walking directly towards the acacia host-tree?

Only the central-place forager *P. gracilis* and the long-distance traveler *P. spinicola* ants walked in an angle directly pointing towards the acacia tree (located at 0°), i.e., their mean walking angle was not different from zero in these two species (Rayleigh test, control $R = 0.02$, $p = 0.46$, rotated $R = 0.02$, $p = 0.44$; control $R = 0.06$, $p = 0.63$, rotated $R = 0.16$, $p = 0.15$, respectively). In the two other in-nest foraging species, workers in both control and treatment discs had a mean walking angle different from zero: *P. nigrocinctus* (Rayleigh test, control $R = 0.97$, $p < 0.0001$, rotated $R = 0.89$, $p < 0.0001$) and *P. flavicornis* (Rayleigh test, control $R = 0.95$, $p < 0.0001$, rotated $R = 0.88$, $p < 0.0001$). Surprisingly again, workers of the central-place forager *P. nigropilosus* did not walked in an angles pointing towards the acacia tree (Rayleigh test, control $R = 0.90$, $p < 0.0001$, rotated $R = 0.89$, $p < 0.0001$).

Walking away from tree on disc edge.

When workers arrived to the edge of the disc, they could walk towards the acacia tree or in the opposite direction (Fig. 4.4g). When comparing among species, the effect of treatment on the frequency of turning away from the acacia tree did not depend on type of foraging (central-place vs. in-nest foragers; $z = 0.99$, $p = 0.32$), but it does depend on

whether ants walked short or long distances away from the tree (interaction of treatment vs. traveling distance, $z = -2.01$, $p = 0.04$). We found that once on the edge of the disc, only in-nest foragers of *P. spinicola* workers were more likely to walk away from the acacia tree when the disc was rotated compared to the control (Fig. 4.4c; Mann-Whitney $U = 379.5$, $p = 0.002$). Rotating the disc had no effect on this behavior in any of the other species (*P. flavicornis*, Fig. 4b, Mann-Whitney $U = 611$, $p = 0.27$; *P. nigrocinctus,* Fig. 4.4a, Mann-Whitney $U = 199.5$, $p = 0.22$; *P. nigropilosus,* Fig. 4.4d, Mann-Whitney $U = 209$, $p = 0.82$; *P. gracilis*, Fig. 4.4e, Mann-Whitney $U = 58$, $p = 0.30$).

Getting off the experimental disc

Once on the edge of the disc, ants could leave the disc by the same point from which they entered or by a different point (Fig. 4.5g). However, rotating the disc similarly affects in-nest foragers and central-place foragers (interaction term of treatment and foraging type: $z = 0.011$, $p = 0.99$), and between short- and long-distance travelers (interaction of treatment and traveled distance: $z = 0.013$, $p = 0.99$). When analyzed by species, in two of the in-nest foragers, *P. nigrocinctus* and *P. spinicola,* rotating the disc often caused more workers to leave the discs by a new point rather by than entrance point when compared to the control (*P. nigrocinctus* Fig. 4.5a; $X^2 = 5.06$, d.f. = 1, $p = 0.025$; *P. spinicola*, Fig. 4.5c; $X^2 = 3.22$, d.f. = 1, $p = 0.072$). This was not observed in the short-distance traveler *P. flavicornis* (Fig. 4.5b; $X^2 = 0.89$, d.f. = 1, $p = 0.34$). For the central-place foragers, the probability of leaving the disc by the entrance point was not affected by the treatment of rotating the disc in *P. nigropilosus* (Fig. 4.5d; $X^2 = 0.71$, d.f. = 1, $p = 0.40$), and *P. gracilis* was the only species where workers left the disc by the entrance point in all trials (Fig. 4.5e).

Time returning to the nest.

Combining all species, the effect of the disc rotation on the time to return to the nest (difference between control and rotated disc) was similar between central-place foragers and in-nest foragers (interaction of foraging type with treatment, $F_{1,66} = 2.08$, $p = 0.15$) and between short- and long-distance travelers (interaction of treatment and traveled distance: $z = 0.16$, $p = 0.68$). When analyzing by species, workers of only one of the two central place foragers *P. gracilis* returned to the host-tree in about the same time in control and rotation treatment (Fig. 4.6e; Mann-Whitney $U = 77$, $p = 0.54$), whereas workers of *P. nigropilosus* ants tended to take longer in the rotation treatment than in controls (Fig. 4.6d; Mann-Whitney $U = 53.5$, $p = 0.06$). All workers of in-nest foraging species on rotated treatments took longer than control ants to return (*P. nigrocinctus:* Fig. 4.6a, Mann-Whitney $U = 59$, $p = 0.0002$; *P. flavicornis:* Fig. 4.6b, Mann-Whitney $U = 132$, $p = 0.04$; and *P. spinicola:* Fig. 6c, Mann-Whitney $U = 62.5$, $p = 0.003$).

Walking speed

Overall, disc rotation effects were not dependent on whether ants were central-place foragers (interaction of foraging type and treatment, $F_{1,165} = 0.54$, $p = 0.46$), or whether they walked long or short-distances away from the host tree (interaction of treatment and traveling distance, $F_{1,165} = 0.68$, $p = 0.40$). Analyzing by species, only an in-nest foraging species, *P. nigrocinctus*, walked more slowly on rotated disc than on control discs (Fig. 4.7a; Mann-Whitney $U = 278$, $p = 0.013$), whereas the other two acacia mutualists did not (*P. flavicornis*, Fig. 4.7b; Mann-Whitney $U = 229$, $p = 0.61$, $n=41$; and *P. spinicola*, Fig. 4.7c; Mann-Whitney $U = 172$, $p = 0.52$). The two central-place foragers did not walk more slowly either (*P. nigropilosus*, Fig. 4.7d Mann-Whitney $U = 87$, $p = 0.78$; and *P. gracilis*, Fig. 4.7e, Mann-Whitney $U = 98.5$, $p = 0.42$).

Returning to disc center

The effect of the disorientation test on the behavior of walking back to the disc center depended on foraging type (interaction of treatment vs. foraging type, $Z = 3.16$, $p = 0.001$), and on whether the species was a short- or long-distance traveler (interaction term of treatment vs. travelled distance, $Z = 3.16$, $p = 0.03$). Workers from all three in-nest forager species more often walked back to the center of the disc when the disc was rotated than when it was in the original position (*P. flavicornis*, Fig. 4.8b, $X^2 = 6.93$, d.f = 1, $p = 0.008$; *P. spinicola*, Fig. 4.8c, $X^2 = 10.20$, d.f. = 1, $p = 0.001$; and *P. nigrocinctus*, Fig. 4.8a, $X^2 = 9.38$, d.f. = 1, $p = 0.002$). This increment in the number of times that workers walked towards the center of the disc or away from the acacia was absent in both central-place foragers (*P. nigropilosus*, Fig. 4.8d; $X^2 = 2.01$, d.f. = 1, $p = 0.15$; and *P. gracilis*, Fig. 4.8e, $X^2 = 0.32$, d.f. = 1, $p = 0.57$).

Spin

The effect of treatment depended on the type of foraging because it increases in in-nest foragers but decreases in central-place foragers (interaction of foraging type with treatment, $Z = 1.95$, $p = 0.050$), but not on whether ants were short-distance or long-distance travelers (interaction of foraging type with treatment, $Z = 1.58$, $p = 0.11$). However, when analyzed by species, workers of any species spun similarly on rotated or control discs (*P. nigrocinctus*, Fig. 4.9a; Mann-Whitney $U = 233$, $p = 0.59$; *P. flavicornis*, Fig. 4.9b; Mann-Whitney $U = 640$, $p = 0.32$; *P. spinicola*, Fig. 4.9c; Mann-Whitney $U = 609.5$, $p = 0.65$; *P. nigropilosus*, Fig. 4.9d; Mann-Whitney $U = 169$, $p = 0.13$; *P. gracilis,* Fig. 4.9e; Mann-Whitney $U = 65.5$, $p = 0.82$).

Stop

The effect of disc rotation on the number of stops by workers was not dependent on type of foraging (interaction of foraging type and treatment, $Z = 1.05$ $p = 0.29$) or on the traveling distance (interaction of treatment and traveling distance, $Z = 0.945$, $p = 0.34$). The results are consistent when analyzed by species: workers of any species on rotated treatments stopped as much as control workers (*P. nigrocinctus,* Fig. 4.10a; Mann-Whitney $U= 206$, $p = 0.21$; *P. flavicornis,* Fig. 4.10b; Mann-Whitney $U= 694$, $p = 0.94$; *P. spinicola,* Fig. 4.10c; Mann-Whitney $U= 628.5$, $p = 0.98$; *P. nigropilosus,* Fig. 4.10d; Mann-Whitney $U= 181.5$, $p = 0.52$; *P. gracilis,* Fig. 4.10e; Mann-Whitney $U= 57$, $p = 0.46$).

DISCUSSION

Obligatory nesting on acacia trees caused the evolution of several aspects of the workers' behavior compared to other species in the genus *Pseudomyrmex*. For example, acacia ant workers are aggressive instead of shy, prune neighboring vegetation instead of ignoring it, and have nocturnal activity instead of being strictly diurnal (Janzen 1966). The cognitive abilities of these workers are also likely to be affected by the association with the plant. We first tested whether central-place foragers were better at navigating toward their nest compared to in-nest foragers. Second, we tested whether performance at the orientation assay was related to the distance workers typically travel away from the nest (long-distance vs. short distance).

Overall, only one of the measured behaviors (returning to the disc center) was consistently associated with foraging mode, with all in-nest foragers and none of the central-place foragers performing the behavior more often when the disc was rotated. For the other behaviors, the pattern was not consistent across foraging modes but there were clear differences between species in their efficiency at performing the orientation task.

Specifically, two species behaved just as predicted according to their foraging strategy and distance traveled away from the host tree: *P. nigrocinctus* and *P. gracilis,* which are a in-nest and central-place forager species respectively. In contrast, *P. flavicornis* workers showed very few behavioral differences among rotated and control discs despite being in-site foragers and to travel short-distances away from the host tree. Also, *P. nigropilosus* workers were more affected by the disorientation assay than expected for a central-place forager, when compared to the other species.

The behavior of returning to the disc center after heading in the wrong direction on rotated treatments increased only in mutualistic in-nest foragers when compared to control discs. Returning to a familiar landscape is a known orientation strategy used by desert ants, even when requires walking away from the nest (Wystrach et al 2012). Just like acacia ants, desert ants largely rely on vision. However, we lack detailed mechanistic studies showing acacia ants are using vision to orient, and more generally how returning to a familiar location helps acacia ants to orient to their nest.

Workers of *P. flavicornis* –one of the in-nest foraging species that travels short-distances from host trees– were clearly less affected by the disorientation test than expected for an in-nest foraging species. Besides the distance workers walk away from the acacia tree to kill vegetation, there could be another ecological aspect in which orienting is more relevant for *P. flavicornis* than for the other two acacia mutualists. Besides the distance workers walk away from the acacia tree to kill vegetation, there could be another ecological aspect in which orienting is more relevant for *P. flavicornis* than for the other two acacia mutualists. Acacia ants may fall off branches or drop themselves when disturbance is occurring on the base of the tree (SAV pers. obs.), and it is possible that *P. flavicornis* workers perform this behavior more often than workers of

P. nigrocinctus or *P. spinicola*. Such dropping-escape strategies could increase the selective pressure for orientating back to the tree, but this hypothesis remains to be tested.

The mechanisms used during navigation in acacia ants have not been studied to date, but research on other ants indicate that workers can use path integration, terrestrial or celestial cues, and systematic search to orient (Cheng et al 2014). As the distance and location of the nest and food reward did not change during the experiment, using path integration would have lead workers directly to the tree after locating the food. Our data suggests that none of the studied species uses path integration for navigation, because even *P. gracilis* workers – which did not show any behavioral difference between control and rotated treatments – started walking in a different direction before heading toward the acacia (control and rotated ants differ in the mean walking angle 3 s after picking up the food). Also, only the central-place forager *P. gracilis* ants spun (i.e., performed 360° turns with no displacement) several times before heading towards the acacia tree in both control and rotated treatments; this behavior was seldom observed in the other species including the sister species *P. nigropilosus*. Spinning may allow *P. gracilis* ants to identify landscape cues to orient towards the acacia tree. While our experiments were not designed to specifically address the navigational mechanisms used by acacia ants to orient, they suggest these mechanisms may differ between closely related species.

The comparisons that we have presented are limited to two groups within the ant subfamily Pseudomyrmicinae (*P. gracilis* group and *P. ferrugineus* group), and should be expanded to include other obligatory plant-associates and closely related central-place foragers. One of the limitations of comparing navigational skills between species is successfully applying a standard test to all of them. Also, species can show unique behaviors that have no equivalent in other species, which makes difficult to score behaviors across species. A strength of our study is that we were able to minimize the

influence of other factors in our comparison by having species of the same genus, naturally nesting on the swollen spines of acacia trees, feeding on nectar and food bodies (all but one species) produced by the tree, and occurring in the same field site. We tried to include *P. viduus* (*P. viduus* group, Ward and Downie 2005) nesting on *Triplaris melanoceras* (Polygonaceae) trees in our study, but workers did not walk or patrol on the lower parts of the tree trunk or on the ground, and they were too shy to get in the experimental disc on a reasonable time. Nonetheless, species in protective plant mutualisms from other ant genera, that also kill the vegetation surrounding the host tree, could be used to evaluate, in another phylogenetic group, the hypothesis that being associated with a tree may affect the orientation abilities of workers.

Conclusion

Navigation in animals has been widely studied in species that evolved remarkable orientation skills (e.g., migratory or food-storing animals). Foraging mode is one of the forces that mostly influences spatial memory and navigational skills in animals (Collett et al 2013). Accordingly, our results suggest that the change in foraging mode, from central-place to in-nest foraging, caused by the mutualisms with a plant could have diminished the navigational skills on acacia ant workers, although this effect was not consistent across species. Ants in obligatory mutualisms with plants present a unique opportunity to study how ecological factors can relax selection for behavioral traits required for orientation in animals.

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TABLES

Species	**P. nc**	**P. fl**	**P. sp**	**P. np**	**P. gr**
Association with acacia tree	Obligate mutualists			Obligate parasite	Facultative parasite
Foraging mode	In-nest			Central-place foraging	
Distance workers travel away from host tree	short	short	long	long	long
Walking angle of first 3 seconds	yes	no	no	yes	yes
Walking angle entire pathway	yes	no	no	yes	no
Walking at an angle pointing towards the acacia	no	no	yes	no	yes
Walking away from tree on disc edge	no	no	yes	no	no
Walking off disc at a point other than the initial entrance point[1]	yes	no	yes*	no	no
Time returning to the nest	yes	yes	yes	yes*	no
Average speed	yes	no	no	no	no
Walking towards disc center	yes	yes	yes	no	no
Number of spins[1]	no	no	no	no	no
Number of stops[1]	no	no	no	no	no

[1]Traits that could not be tested for phylogenetic signal on the effect size of control vs rotation treatment.

Table 4. 1. Summary of behavioral differences between ant workers in control and rotation treatments of the disorientation tests. We sampled workers of five species of *Pseudomyrmex* ants that differed in their association with acacia trees, which in turn determines the foraging mode. We expected control and treatment workers of central place foragers to differ in fewer of the assessed behaviors than workers of in-nest foraging species. Among in-nest foragers, we expected more behaviors to differ between control and treatment workers the least workers travel away from their host tree to kill neighboring vegetation. Shaded cells highlight results suggesting that workers were disoriented.

Trait	Phylogenetic signal	P-value
Walking away from tree on disc edge	0.24	0.65
Time returning to the nest	0.35	0.45
Average speed	0.59	0.28
Returning to disc center	0.24	0.65
Mean walking angle of entire pathway*	1.36	0.50

Table 4. 2. Tests for phylogenetic signal in the effect size of control vs. rotation treatments on various behavioral trait (Blomberg K or *D-value), and P-value of testing for a significant deviation from random expectations (randomization test, see Methods). D-values of one, as well as K-values larger than one, indicate phylogenetic signal on the trait. Because none of the test statistics approached these values, behavioral differences between control vs. rotation treatments were not confounded by phylogenetic signal.

FIGURES

Figure 4. 1. Acacia ant species vary in the area around the host tree that workers clear of other vegetation. **(a)** *Pseudomyrmex nigrocinctus* and **(b)** *P. flavicornis* prune vegetation in a radius about five times smaller than clearings of **c)** *P. spinicola*.

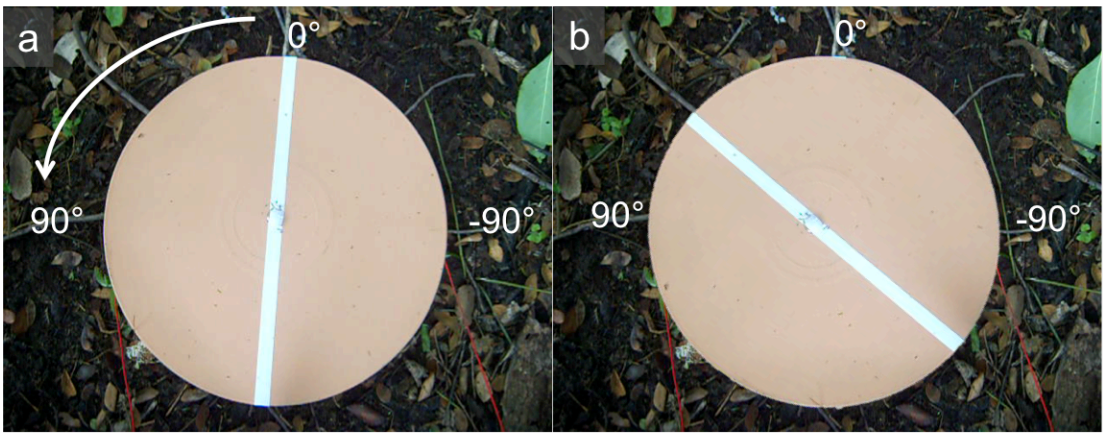

Figure 4. 2. Experimental set up used in disorientation tests. A rotary disc was placed on the ground next to a acacia host-tree inhabited by a colony of one of the five tested *Pseudomyrmex* species. (**a**) Workers walked on the disc at the point nearest to the acacia tree (0° orientation), and picked up a food item placed on the center of the disc. (**b**) While the worker was picking up the food, the disc was rotated 45° clockwise (or counterclockwise, not shown) by pulling the red strings. As control treatment, the disc was rotated 20° in one direction and then 20° on the opposite direction to return the disc back to the initial 0° orientation, to control for the effect of disc movement.

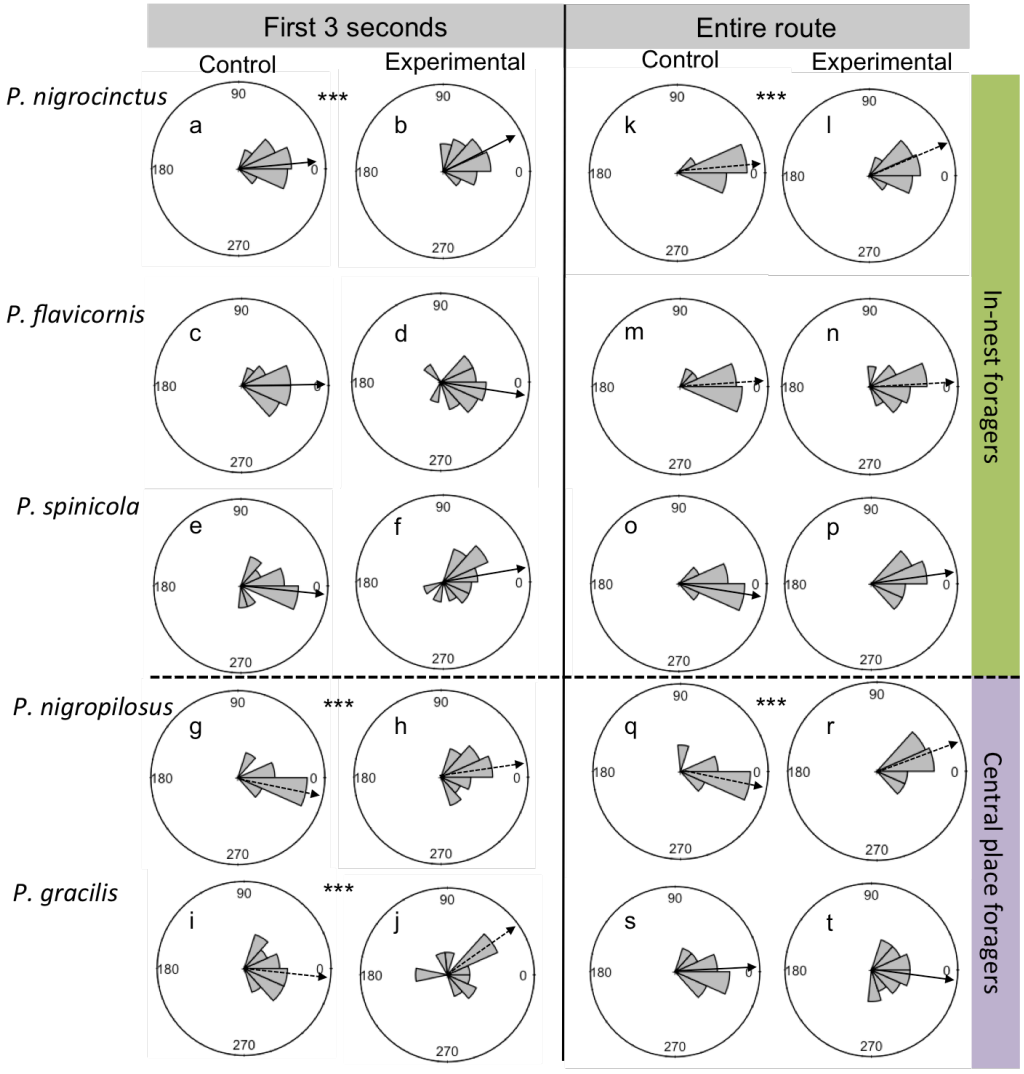

Figure 4. 3. Histograms of walking angles relative to the orientation towards the nest (acacia host-tree at 0°) calculated during the first 3.3 seconds after a worker picked up a food item in control and rotation treatments (**a**-**j**) or calculated at the point where the worker walked off the experimental disc (**k-t**). *Pseudomyrmex nigrocinctus, P. flavicornis* and *P. spinicola* have a mutualistic association with acacia trees, and are all so-called in-nest foragers. Central-place foraging ant species include *Pseudomyrmex nigropilosus*, an acacia parasite that also extracts food from nearby acacia trees; and *Pseudomyrmex gracilis*, a species that nests on acacia trees and preys on small arthropods in the forest canopy. The arrow in each circle denotes the mean (continuous line, for normally distributed data) or the median angle (dashed line, for non-normally distributed data), and asterisks denote significant differences in ant behavior in control and rotated discs.

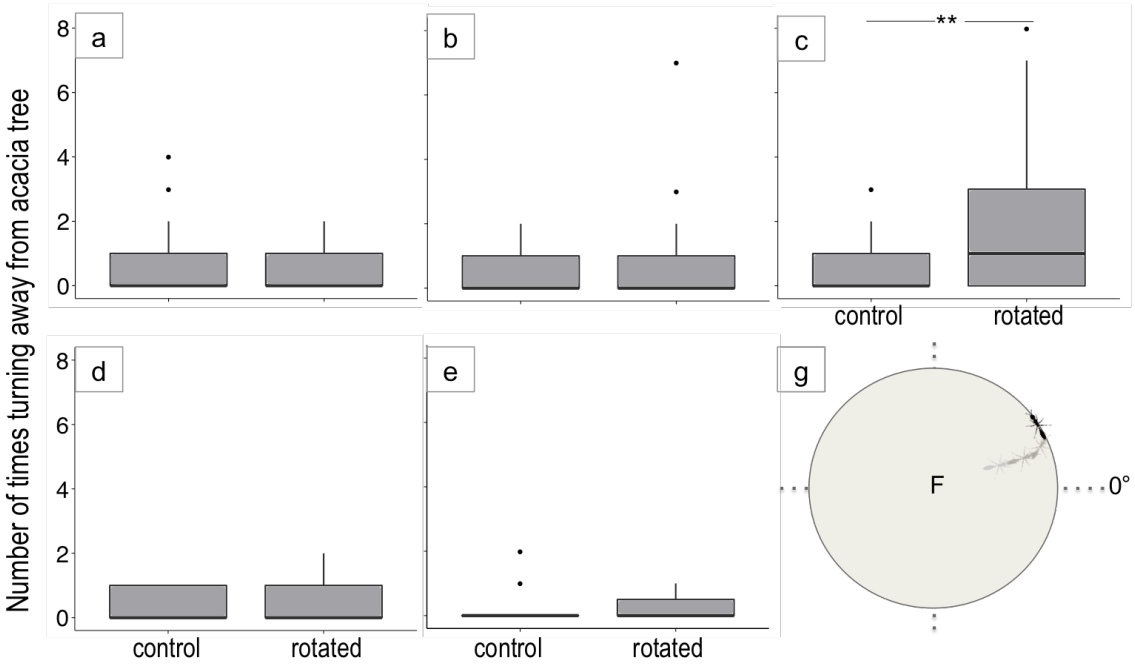

Figure 4. 4. Number of times that ants walked in opposite direction from their acacia host-tree on the edge of the experimental disc, after picking up the food reward in control and rotation treatments. Workers on rotation treatments turned away from the host-tree on the disc edge more often than workers on control treatments in the acacia mutualists *P. spinicola* (c), but not in the within-nest foraging *P. nigrocinctus* (a) or *P. flavicornis* (b), nor in the central place foragers *P. nigropilosus* (d) and *P. gracilis* (e). Median, interquartile range (IQR), 1.5*IQR, and outliers are represented by the dark horizontal line, box, whisker and dots respectively. Statistically significant differences are shown with asterisks ($p<0.05$). (g) The inset shows how a worker might get to the edge of the disc and turn away from its nesting tree (located at 0°), after picking up the food (F).

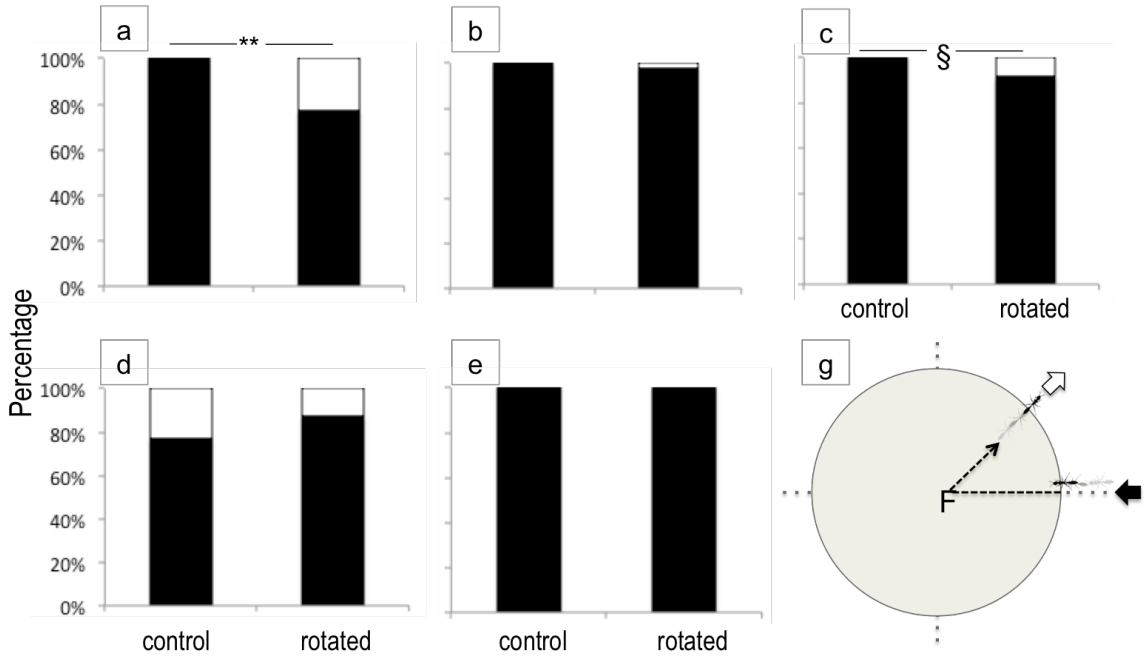

Figure 4. 5. Number of times that ants exited the experimental control or rotated discs at the entrance point (black bars), or at a different point (white bars), for workers of the three in-nest foraging ant species *P. nigrocinctus* (**a**), *P. flavicornis* (**b**) and *P. spinicola* (**c**), and the central-place foraging species *P. nigropilosus* (**d**) and *P. gracilis* (**e**). The diagram (**g**) depicts how a worker might enter the disc at one point (black arrow, closer to the acacia tree at 0°), but leave it at a different point (white arrow) after picking up a food item placed at the disc center (F). Statistically significant differences between control and rotation treatments are shown (** $p<0.05$; § $0.05 < p < 0.10$).

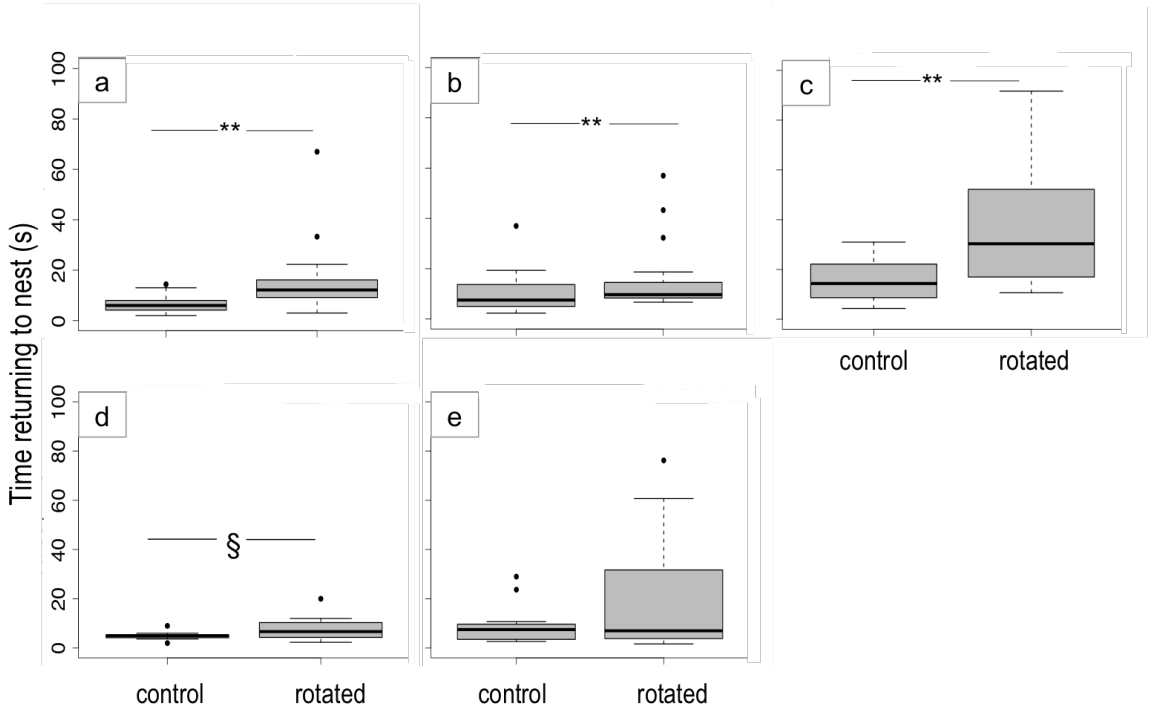

Figure 4. 6. Time to return to the nest after picking up the food reward in control and rotated treatments. Workers took longer to return to the nest on the rotated treatment than on the control treatment in the acacia mutualists (**a**) *P. nigrocinctus* , (**b**) *P. flavicornis*, and (**c**) *P. spinicola* . This difference was marginally significant on the central-place forager *P. nigropilosus* (**c**). The rotation treatment had no effect on the time to return to the disc for the central-place foragers of *P. gracilis* (**e**). Median, interquartile range (IQR), 1.5*IQR, and outliers are represented by the dark horizontal line, box, whisker and dots respectively. Statistically significant differences between control and treatment (rotated disc) are shown (** $p<0.05$; § $0.05 < p < 0.10$).

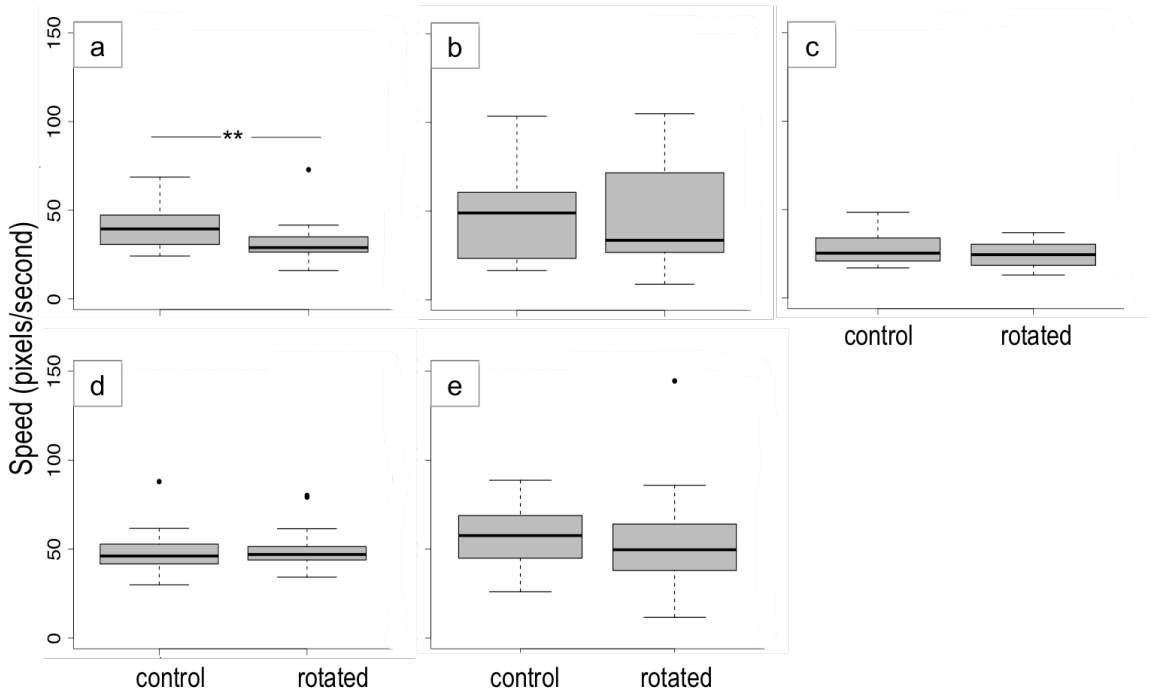

Figure 4. 7. Walking speed (pixels/s) of workers returning to the nest after picking up the food reward in control and rotation treatments. Workers on rotation treatments walked more slowly than workers on control treatments in the acacia mutualists *P. nigrocinctus* (**a**). The treatment had no effect on the walking speed of the other two mutualistic species, *P. flavicornis* (**b**), and *P. spinicola* (**c**), or in the two central-place foragers *P. nigropilosus* (**c**) or *P. gracilis* (**e**). Median, interquartile range (IQR), 1.5*IQR, and outliers are represented by the dark horizontal line, box, whisker and dots respectively. Statistically significant differences between control and treatment (rotated disc) are shown (** $p < 0.05$).

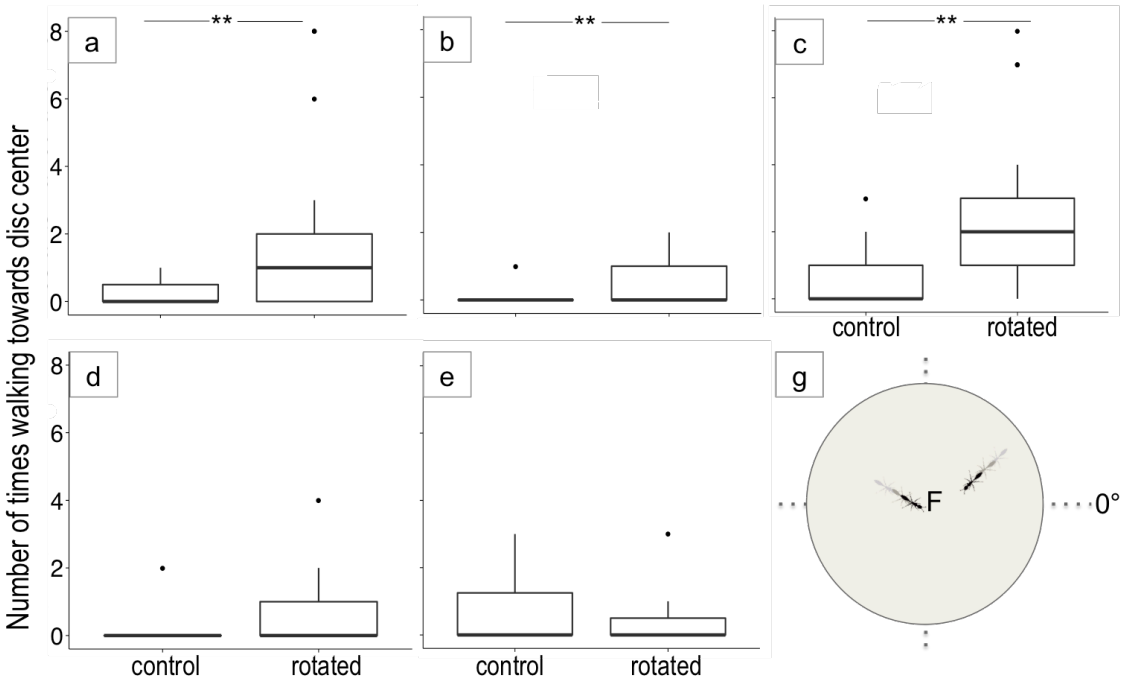

Figure 4. 8. Number of times that ants walked towards the center of the disc according to the treatment, for workers of the in-nest foragers: *P. nigrocinctus* (**a**), *P. flavicornis* (**b**) and *P. spinicola* (**c**); and the central-place foragers *P. nigropilosus* (**d**) and *P. gracilis* (**e**). The diagram (**g**) represents the experimental disc where ants were tested, and the ant silhouettes depicts how two different workers walk toward the disc center after picking up the food (located at **F**); the acacia tree would be at 0°. Statistically significant differences between control and treatment (rotated disc) are shown (** $p<0.05$; § $0.05 > p > 0.10$).

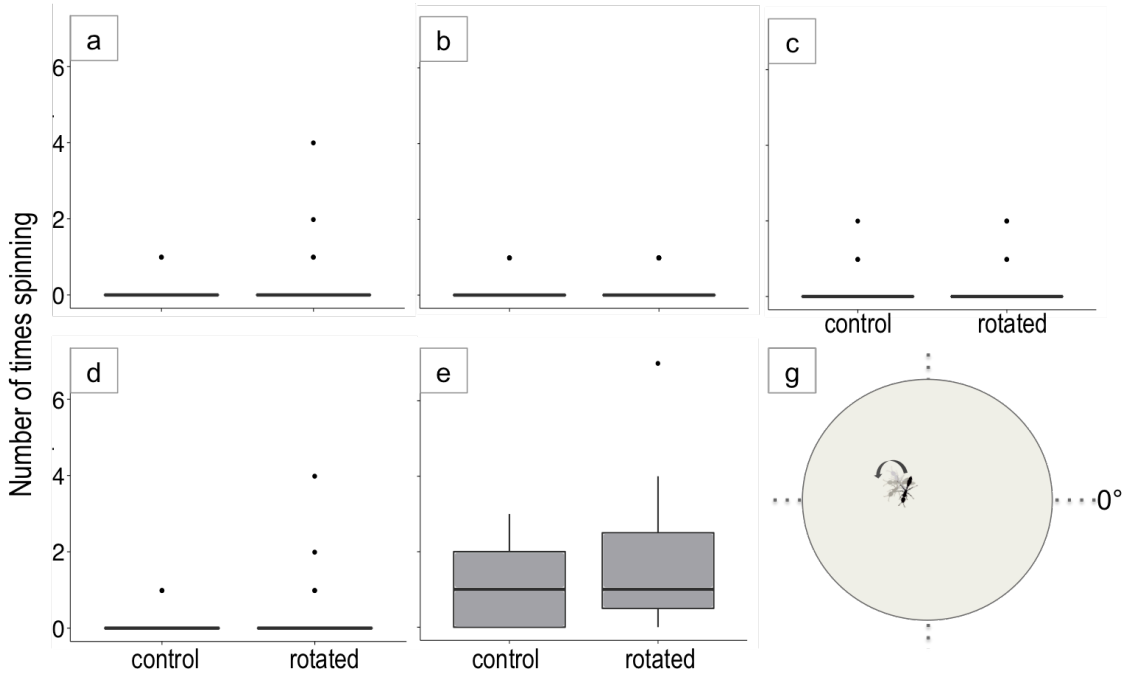

Figure 4. 9. Number of spins (360° turns without displacement) that ants performed after picking up the food item. Workers on rotation treatments were not spinning more than workers on control treatments for any species: *P. nigrocinctus* (**a**), *P. flavicornis* (**b**), *P. spinicola* (**c**), the robber *P. nigropilosus* (**d**) and the predator *P. gracilis* (**e**). The diagram (**g**) represents the experimental disc where ants were tested, and the ant silhouette shows how an ant might perform a spin after picking up the food (located at **F**); the acacia tree would be at 0°.

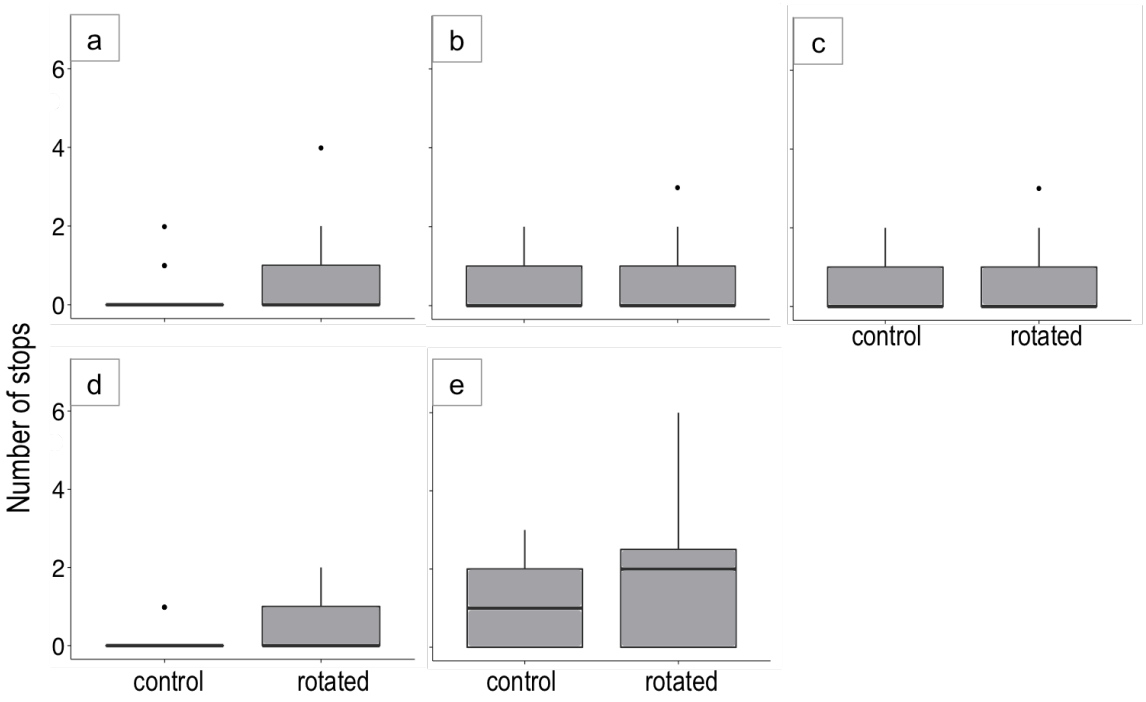

Figure 4. 10. Number of stops that ants performed after picking up the food item. Rotating the disc did not increase this behavior in the in-nest foraging species: (**a**) *P. nigrocinctus*, (**b**) *P. flavicornis*, and (**c**) *P. spinicola*; or in the central-place foraging species (**d**) *P. nigropilosus* or (**e**) *P. gracilis*.

References

Adolphs R (2001) The neurobiology of social cognition. Current Opinion in Neurobiology 11:231–239.

Agostinelli C, Lund U (2013) R package "circular": Circular Statistics.

Amador-Vargas S (2008) Spartan defense in the Thermopylae pass: Strategic defense by aggregations of *Pseudomyrmex spinicola* (Hymenoptera, Formicidae) on the trunk of *Acacia collinsii* (Mimosaceae). Insectes Sociaux 55:241–245.

Amador-Vargas S (2012a) Plant killing by mutualistic ants increases the density of host species seedlings in the dry forest of Costa Rica. Psyche 2012:28–33.

Amador-Vargas S (2012b) Behavioral responses of acacia ants correlate with age and location on the host plant. Insectes Sociaux 59:341–350.

Amador-Vargas S (2012c) Run, robber, run: parasitic acacia ants use speed and evasion to steal food from ant-defended trees. Physiological Entomology 37:323–329.

Anderson C, McShea DW (2001) Individual versus social complexity, with particular reference to ant colonies. Biological Reviews 76:211–237.

Archetti M, Úbeda F, Fudenberg D, et al (2011) Let the right one in: A microeconomic approach to partner choice in mutualisms. The American Naturalist 177:75–85.

Asher CL, Nascimento FS, Sumner S, Hughes WO (2013) Division of labour and risk taking in the dinosaur ant, *Dinoponera quadriceps* (Hymenoptera: Formicidae). Myrmecological news18:121–129.

Balda RP, Kamil AC (1989) A comparative study of cache recovery by three corvid species. Animal Behaviour 38:486–495.

Bauer S, Böhm M, Witte V, Foitzik S (2009) An ant social parasite in-between two chemical disparate host species. Evolutionary Ecology 24:317–332.

Bednekoff PA, Balda RP, Kamil AC, Hile AG (1997) Long-term spatial memory in four seed-caching corvid species. Animal Behaviour 53:335–341.

Beekman M, Ratnieks FLW (2000) Long-range foraging by the honey-bee, *Apis mellifera* L. Functional Ecology 14:490–496.

Blomberg SP, Garland T Jr, Ives AR (2003) Testing for phylogenetic signal in comparative data: behavioral traits are more labile. Evolution 57: 717–745.

Blumstein D, Evans C, Daniels J (2006) JWatcher 1.0. See http://www. jwatcher. ucla. edu

Bonner JT (2004) Perspective: The size-complexity rule. Evolution 58: 1883–1890.

Bourke (1999) Colony size, social complexity and reproductive conflict in social insects. Journal of Evolutionary Biology 12: 245–257.

Bourke AFG (2011) Principles of Social Evolution. Oxford

Bourke AFG, Franks NR (1995) Social evolution in ants. Princeton University Press

Breed MD, Cook C, Krasnec MO (2012) Cleptobiosis in social insects. Psyche: A Journal of Entomology 2012: 1–7.

Bronstein JL (1998) The contribution of ant-plant protection studies to our understanding of mutualism. Biotropica 30: 150–161.

Brown D (2014) Tracker 4.86. https://www.cabrillo.edu/~dbrown/tracker/

Byrne RW, Bates LA (2007) Sociality, evolution and cognition. Current Biology 17:R714–R723.

Canty A, Ripley B (2014) boot: Bootstrap R (S-Plus).

Chamberlain SA, Holland JN (2009) Quantitative synthesis of context dependency in ant–plant protection mutualisms. Ecology 90:2384–2392.

Cheng K, Schultheiss P, Schwarz S, et al (2014) Beginnings of a synthetic approach to desert ant navigation. Behavioural Processes 102: 51–61.

Clement L, Köppen S, Brand W, Heil M (2008) Strategies of a parasite of the ant–acacia mutualism. Behavioral Ecology and Sociobiology 62:953–962.

Collett M, Chittka L, Collett TS (2013) Spatial memory in insect navigation. Current Biology 23:R789–R800

Cronin G (1998) Between-species and temporal variation in acacia-ant-herbivore interactions. Biotropica 30:135–139.

Dabney A, Storey JD (2004) qvalue: Q-value estimation for false discovery rate control.

Davidson DW (1993) Evolutionary ecology of symbiotic ant-plant relationships. International Society of Hymenopterists 2:13.

Davidson DW, Longino JT, Snelling RR (1988) Pruning of host plant neighbors by ants: an experimental approach. Ecology 69:801–808.

Dornhaus A, Holley J-A, Franks NR (2009) Larger colonies do not have more specialized workers in the ant *Temnothorax albipennis*. Behavioral Ecology 20:922 –929.

Dunbar R (2003) The social brain: mind, language, and society in evolutionary perspective. Annual Review of Anthropology 32:163–181.

Dunbar RIM (1998) The social brain hypothesis. Evol Anthropol 6:178–190.

Dunbar RIM, Shultz S (2007) Evolution in the social brain. Science 317:1344–1347.

Eberhard WG, Wcislo WT (2011) Grade changes in brain-body allometry: morphological and behavioral correlates of brain size in miniature spiders, insects and other invertebrates. Adv Ins Physiol 40:155–214.

Farris SM, Robinson GE, Fahrbach SE (2001) Experience- and age-related outgrowth of intrinsic neurons in the mushroom bodies of the adult worker honeybee. J Neurosci 21:6395–6404.

Farris SM, Schulmeister S (2011) Parasitoidism, not sociality, is associated with the evolution of elaborate mushroom bodies in the brains of hymenopteran insects. Proceedings of the Royal Society B: Biological Sciences 1707:940–51.

Federle W, Maschwitz U, Holldöbler B (2002) Pruning of host plant neighbours as defence against enemy ant invasions: *Crematogaster* ant partners of *Macaranga* protected by "wax barriers" prune less than their congeners. Oecologia 132:264–270.

Fiala B, Maschwitz U, Pong TY, Helbig AJ (1989) Studies of a South East Asian ant-plant association: protection of *Macaranga* trees by *Crematogaster borneensis*. Oecologia 79:463–470.

Fiala JC (2005) Reconstruct: a free editor for serial section microscopy. Journal of Microscopy 218:52–61.

Fonseca CR (1993) Nesting space limits colony size of the plant-ant *Pseudomyrmex concolor*. Oikos 67:473–482.

Fonseca CR (1999) Amazonian ant-plant interactions and the nesting space limitation hypothesis. Journal of Tropical Ecology 15:807–825.

Frederickson ME (2005) Ant species confer different partner benefits on two neotropical myrmecophytes. Oecologia 143:387–395.

Frederickson ME, Greene MJ, Gordon DM (2005) "Devil"s gardens' bedevilled by ants. Nature 437:495–496.

Fritz SA, Purvis A (2010) Selectivity in mammalian extinction risk and threat types: a new measure of phylogenetic signal strength in binary traits. Conservation Biology 24:1042–1051.

Gautrais J, Theraulaz G, Deneubourg J-L, Anderson C (2002) Emergent polyethism as a consequence of increased colony size in insect societies. J Theor Biol 215:363–373.

Giraldo YM, Patel E, Gronenberg W, Traniello JFA (2013) Division of labor and structural plasticity in an extrinsic serotonergic mushroom body neuron in the ant *Pheidole dentata*. Neuroscience Letters 534:107–111.

Gómez-Acevedo S, Rico-Arce L, Delgado-Salinas A, et al (2010) Neotropical mutualism between *Acacia* and *Pseudomyrmex*: Phylogeny and divergence times. Mol Phylogenet Evol. 56: 393-408.

Gronenberg W (2008) Structure and function of ant (Hymenoptera: Formicidae) brains: Strength in numbers. Myrmecological News 11:25–36.

Gronenberg W (2001) Subdivisions of hymenopteran mushroom body calyces by their afferent supply. The Journal of Comparative Neurology 435:474–489.

Gronenberg W, Heeren S, Holldöbler B (1996) Age-dependent and task-related morphological changes in the brain and the mushroom bodies of the ant Camponotus floridanus. J Exp Biol 199: 2011–2019.

Gronenberg W, Paul J, Just S, Hölldobler B (1997) Mandible muscle fibers in ants: fast or powerful? Cell and Tissue Research 289:347–361.

Gronenberg W, Riveros A (2009) Social brains and behavior—past and present. Organization of insect societies: from genome to sociocomplexity, Gadau J., Fewell J. Harvard University Press, pp 377–401

Hanson PE, Longino JT (2006) Hormigas. Hymenoptera de la región neotropical, Hanson, P. & Gauld, I. American Entomological Institute, p 994

Hardin JW, Hilbe JM (2012) Generalized Estimating Equations, Second Edition. CRC Press

Heil M, McKey D (2003) Protective ant-plant interactions as model systems in ecological and evolutionary research. Annu Rev Ecol Evol Syst 34:425–553.

Heisenberg M (1998) What do the mushroom bodies do for the insect brain? An introduction. Learn Mem 5:1–10

Herron MD, Michod RE (2008) Evolution of complexity in the Volvocine algae: transitions in individuality through Darwin's eye. Evolution 62:436–451.

Højsgaard S, Yan J, Halekoh U (2005) The R Package geepack for Generalized Estimating Equations. Journal of Statistical Software 15:1–11.

Holbrook CT, Barden PM, Fewell JH (2011) Division of labor increases with colony size in the harvester ant *Pogonomyrmex californicus*. Behavioral Ecology 22:960 – 966.

Holldöbler B (1983) Territorial behavior in the green tree ant (*Oecophylla smaragdina*). Biotropica 15:241–250. doi: 10.2307/2387648

Hölldobler B, Wilson EO (1990) The ants. Harvard University Press

Iyengar EV (2008) Kleptoparasitic interactions throughout the animal kingdom and a re-evaluation, based on participant mobility, of the conditions promoting the evolution of kleptoparasitism. Biological Journal of the Linnean Society 93:745–762.

Jackson RR, Salm K, Pollard SD (2008) Snatching prey from the mandibles of ants, a feeding tactic adopted by East African jumping spiders. Journal of Arachnology 36:609–611.

Jaffe K, Perez E (1989) Comparative study of brain morphology in ants. Brain Behav Evol 33:25–33.

Janzen DH (1991) *Pseudomyrmex ferruginea* (Hormiga del cornizuelo, acacia-ant). Historia Natural de Costa Rica, Janzen, DH. pp 776–778

Janzen DH (1966) Coevolution of mutualism between ants and acacias in Central America. Evolution 20:249–275.

Janzen DH (1969) Allelopathy by myrmecophytes: The ant *Azteca* as an allelopathic agent of *Cecropia*. Ecology 50:147. doi: 10.2307/1934677

Janzen DH (1975) *Pseudomyrmex nigropilosa*: A parasite of a mutualism. Science 188:936–937.

Janzen DH (1974) Swollen-thorn acacias of Central America. Smithsonian Contributions to Botany 13:1–131.

Jeral JM, Breed MD, Hibbard BE (1997) Thief ants have reduced quantities of cuticular compounds in a ponerine ant, *Ectatomma ruidum*. Physiol Entomol 22:207–211.

Krebs CJ (1999) Ecological methodology. Benjamin/Cummings

Kronauer DJC (2004) Trophic parasitism of a wasp (Hymenoptera: Ampulicidae: Ampulex sp.) on the ant *Ectatomma ruidum* (Roger, 1860) (Hymenoptera: Formicidae). Myrmecologische Nachrichten 6:77–78.

LaPierre L, Hespenheide H, Dejean A (2007) Wasps robbing food from ants: a frequent behavior? Naturwissenschaften 94:997–1001.

Larrea-Alcázar DM, Simonetti JA (2007) Why are there few seedlings beneath the myrmecophyte *Triplaris americana*? Acta Oecologica 32:112–118.

Laughlin SB, van Steveninck RRD, Anderson JC (1998) The metabolic cost of neural information. Nat Neurosci 1:36–41. doi: 10.1038/236

Lenoir A, D'Ettorre P, Errard C, Hefetz A (2001) Chemical ecology and social parasitism in ants. Annu Rev Entomol 46:573–599.

Lihoreau M, Latty T, Chittka L (2012) An exploration of the social brain hypothesis in insects. Front Physiol.

Liu L, Wolf R, Ernst R, Heisenberg M (1999) Context generalization in *Drosophila* visual learning requires the mushroom bodies. Nature 400:753–756.

Maguire EA, Gadian DG, Johnsrude IS, et al (2000) Navigation-related structural change in the hippocampi of taxi drivers. PNAS 97:4398–4403.

Maguire EA, Woollett K, Spiers HJ (2006) London taxi drivers and bus drivers: A structural MRI and neuropsychological analysis. Hippocampus 16:1091–1101.

Martin SJ, Jenner EA, Drijfhout FP (2007) Chemical deterrent enables a socially parasitic ant to invade multiple hosts. Proc Biol Sci 274:2717–2722.

Meehan CJ, Olson EJ, Reudink MW, et al (2009) Herbivory in a spider through exploitation of an ant-plant mutualism. Current Biology 19:R892–R893.

Meyers LS, Gamst G, Guarino AJ (2006) Applied Multivariate Research: Design and Interpretation. SAGE

Molina Y, O'Donnell S (2007) Mushroom body volume is related to social aggression and ovary development in the paperwasp *Polistes instabilis*. Brain, Behavior and Evolution 70:137–144.

Morawetz W, Henzl M, Wallnofer B (1992) Tree killing by herbicide producing ants for the establishment of pure *Tococa occidentalis* populations in the Peruvian Amazon. Biodivers Conserv 1:19–33.

Muscedere ML, Traniello JFA (2012) Division of labor in the hyperdiverse ant genus *Pheidole* is associated with distinct subcaste- and age-related patterns of worker brain organization. PLoS ONE 7:e31618.

Nishikawa M, Watanabe H, Yokohari F (2012) Higher brain centers for social tasks in worker ants, *Camponotus japonicus*. J Comp Neurol 520:1584–1598.

Niven JE, Laughlin SB (2008) Energy limitation as a selective pressure on the evolution of sensory systems. J Exp Biol 211:1792–1804.

Orme D (2013) caper: Comparative Analyses of Phylogenetics and Evolution in R.

Pacala SW, Gordon DM, Godfray HCJ (1996) Effects of social group size on information transfer and task allocation. Evolutionary Ecology 10:127–165.

Paul J, Gronenberg W (1999) Optimizing force and velocity: mandible muscle fibre attachments in ants. Journal of Experimental Biology 202: 797 –808.

Pérez-Barbería FJ, Shultz S, Dunbar RIM (2007) Evidence for coevolution of sociality and relative brain size in three orders of mammals. Evolution 61:2811–2821.

Perfecto I, Vandermeer JH (1993) Cleptobiosis in the ant *Ectatomma ruidum* in Nicaragua. Ins Soc 40:295–299.

Raine NE, Willmer P, Stone GN (2002) Spatial structuring and floral avoidance behavior prevent ant–pollinator conflict in a Mexican ant-acacia. Ecology 83:3086–3096.

R Core Team (2013) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria

Renner SS, Ricklefs RE (1998) Herbicidal activity of domatia-inhabiting ants in patches of *Tococa guianensis* and *Clidemia heterophylla*. Biotropica 30:324–327.

Revell LJ (2012) phytools: an R package for phylogenetic comparative biology (and other things). Methods in Ecology and Evolution 3:217–223.

Richard F-J, Dejean A, Lachaud J-P (2004) Sugary food robbing in ants: a case of temporal cleptobiosis. Comptes Rendus Biologies 327:509–517.

Rico-Gray V, Oliveira PS (2007) The Ecology and Evolution of Ant-Plant Interactions. University of Chicago Press

Risch S, McClure M, Vandermeer J, Waltz S (1977) Mutualism between three species of tropical *Piper* (Piperaceae) and their ant inhabitants. American Midland Naturalist 98:433–444.

Riveros AJ, Gronenberg W (2010) Brain allometry and neural plasticity in the bumblebee *Bombus occidentalis*. Brain Behav Evol 75:138–148.

Riveros AJ, Seid MA, Wcislo WT (2012) Evolution of brain size in class-based societies of fungus-growing ants (Attini). Animal Behaviour 83:1043–1049.

Rosumek FB, Silveira FAO, Neves F de S, et al (2009) Ants on plants: a meta-analysis of the role of ants as plant biotic defenses. Oecologia 160:537–549.

Sachs JL, Mueller UG, Wilcox TP, Bull JJ (2004) The evolution of cooperation. The Quarterly Review of Biology 79:135–160.

Savicky P (2009) pspearman: Spearman's rank correlation test.

Schneider CA, Rasband WS, Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. Nat Meth 9:671–675.

Schupp EW (1986) *Azteca* protection of *Cecropia*: ant occupation benefits juvenile trees. Oecologia 70:379–385.

Sendova-Franks A, Franks NR (1993) Task allocation in ant colonies within variable environments (a study of temporal polyethism: Experimental). Bulletin of Mathematical Biology 55:75–96.

Smith AR, Seid MA, Jiménez LC, Wcislo WT (2010) Socially induced brain development in a facultatively eusocial sweat bee *Megalopta genalis* (Halictidae). Proceedings of the Royal Society B: Biological Sciences 277:2157 –2163.

Steck K, Hansson BS, Knaden M (2009) Smells like home: desert ants, *Cataglyphis fortis*, use olfactory landmarks to pinpoint the nest. Frontiers in Zoology 6:5.

Storey JD, Taylor JE, Siegmund D (2004) Strong control, conservative point estimation and simultaneous conservative consistency of false discovery rates: a unified approach. Journal of the Royal Statistical Society: Series B (Statistical Methodology) 66:187–205.

Strausfeld NJ (2012) Arthropod Brains: Evolution, Functional Elegance, and Historical Significance. Belknap Press of Harvard University Press

Strausfeld NJ, Hansen L, Li Y, et al (1998) Evolution, discovery, and interpretations of arthropod mushroom bodies. Learning & Memory 5:11 –37.

Suarez AV, Moraes CD, Ippolito A (1998) Defense of *Acacia collinsii* by an obligate and nonobligate ant species: the significance of encroaching vegetation. Biotropica 30:480–482.

Szathmáry E, Maynard Smith J (1995) The major evolutionary transitions. Nature 374:227–232.

Tang S, Guo A (2001) Choice behavior of *Drosophila* facing contradictory visual cues. Science 294:1543–1547.

Thomas ML, Elgar MA (2003) Colony size affects division of labour in the ponerine ant *Rhytidoponera metallica*. Naturwissenschaften 90:88–92.

Trager MD, Bhotika S, Hostetler JA, et al (2010) Benefits for plants in ant-plant protective mutualisms: A meta-analysis. PLoS ONE 5:e14308.

Ward PS (1990) The ant subfamily Pseudomyrmecinae (Hymenoptera: Formicidae): generic revision and relationship to other formicids. Systematic Entomology 15:449–489.

Ward PS (1993) Systematic studies on *Pseudomyrmex* acacia-ants (Hymenoptera: Formicidae: Pseudomyrmecinae). Journal of Hymenoptera Research 2:117–168.

Ward PS, Downie DA (2005) The ant subfamily Pseudomyrmecinae (Hymenoptera: Formicidae): phylogeny and evolution of big-eyed arboreal ants. Systematic Entomology 30:310–335.

Wehner R, Fukushi T, Isler K (2007) On being small: brain allometry in ants. Brain, Behavior & Evolution 69:220–228.

Wilson EO (1971) The insects societies. Belknap Press of Harvard University Press

Withers GS, Fahrbach SE, Robinson GE (1993) Selective neuroanatomical plasticity and division of labour in the honeybee. Nature 364:238–240.

Witte V, Foitzik S, Hashim R, et al (2009) Fine tuning of social integration by two myrmecophiles of the ponerine army ant, *Leptogenys distinguenda*. Journal of Chemical Ecology 35:355–367.

Wystrach A, Beugnon G, Cheng K (2012) Ants might use different view-matching strategies on and off the route. J Exp Biol 215:44–55.

Yumoto T, Maruhashi T (1999) Pruning behavior and intercolony competition of *Tetraponera* (*Pachysima) aethiops* (Pseudomyrmecinae, Hymenoptera) in *Barteria fistulosa* in a tropical forest, Democratic Republic of Congo. Ecological Research 14:393–404.

Zar JH (2010) Biostatistical Analysis. Prentice Hall

Zurbuchen A, Landert L, Klaiber J, et al (2010) Maximum foraging ranges in solitary bees: only few individuals have the capability to cover long foraging distances. Biological Conservation 143:669–676.

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